

Activation of G-protein coupled receptors targeting gastrointestinal hormone secretion in Type 2 Diabetes

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Summary

G-protein coupled receptors (GPCRs) play an essential role in the ability of a cell to react to its environment. The presence of these receptors on the cell membrane allow the external environment of a cell to affect the intracellular signalling that takes place. For this reason GPCRs are an attractive drug target. Targeting of class B GPCRs through the GLP-1 receptor (GLP1R) agonists is an already approved therapy for the treatment of Type 2 Diabetes. Class A rhodopsin-like GPCRs are currently the most popular drug targets for current FDA approved drugs. GPCRs are also attractive drug targets due to the wide range of ligands that bind to GPCRs such as free fatty acids during digestion.

This thesis examined the effects of activation of both synthetic and endogenous ligands for GPR39, GPR55, GPR75, GPR119 and GPR120 on the secretion of the incretin hormones GLP-1 and GIP. The insulintropic effects of these receptors was also investigated. GLP-1 and GIP secretion were studied using GLUTag and pGIPneo STC-1 cells respectively while insulin secretion was assessed in BRIN-BD11 cells and isolated pancreatic islets. The cytotoxicity of GPCR agonists was determined using MTT assay in GLUTag and pGIPneo STC-1 cells and Alamar blue assay in BRIN-BD11 cells. Membrane integrity was further studied using LDH assay. The expression and cellular localisation of GPCRs and intestinal hormones was determined using qPCR and double immunohistochemistry in both cell lines and small intestine. The acute *in vivo* effects of intestinal GPCR activation on blood glucose as well as plasma GLP-1, GIP and insulin were examined in male Swiss TO mice. The chronic effects biological effects of GPR55 agonist Abn-CBD and GPR119 agonist GPR119 as a monotherapy and combination therapy with the DPP-IV inhibitor Sitagliptin were assessed.

GPCR agonists were able to demonstrate GLP-1, GIP and insulin secretory ability both *in vitro* and *in vivo*. The expression of GPCRs in the L and K cells of the intestine were confirmed using immunohistochemistry. Oral administration of GPCR agonists as a monotherapy or combination therapy resulted increased incretin hormone and insulin secretion as well as reduced blood glucose. The effects of chronic treatment of AS1269574 and Abn-CBD in streptozotocin induced diabetic mice improved glucose homeostasis.

Overall this thesis identified the ability of GPCR ligands to affect glucose homeostasis through the incretin hormone pathway as well as through direct action on the pancreas. The work carried out in this thesis demonstrates that GPCR based therapies have anti-diabetic potential and may be an important therapeutic strategy in the treatment of diabetes in the future.

Abbreviations

Abn-CBD	Abnormal cannabidiol
ALA	α -linolenic acid
AUC	Area under the curve
BSA	Bovine serum albumin
BW	Body weight
Ca²⁺	Calcium
CaCl₂	Calcium chloride
CaCl₂.2H₂O	Calcium chloride dehydrate
cAMP	Cyclic adenosine monophosphate
CB₁	Type 1 cannabinoid receptor
CB₂	Type 2 cannabinoid receptor
CBD	Cannabidiol
CCK	Cholecystokinin
CCL5	Chemokine (C-C motif) ligand 5
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DEXA	Dual energy X-ray absorption
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DPP-IV	Dipeptidyl peptidase-IV
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FFAR	Free fatty acid receptor
FITC	Fluorescein isothiocyanate
GCGR	Glucagon receptor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors

GIP	Glucose-dependent insulintropic polypeptide
GIPR	GIP receptor
GLP-1	Glucagon-like peptide-1
GLP1R	GLP-1 receptor
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GPR4	G-protein coupled receptor 4
GPR38	G-protein coupled receptor 38
GPR39	G-protein coupled receptor 39
GPR40	G-protein coupled receptor 40
GPR41	G-protein coupled receptor 41
GPR43	G-protein coupled receptor 43
GPR55	G-protein coupled receptor 55
GPR68	G-protein coupled receptor 68
GPR75	G-protein coupled receptor 75
GPR84	G-protein coupled receptor 84
GPR119	G-protein coupled receptor 119
GPR120	G-protein coupled receptor 120
GPR132	G-protein coupled receptor 38
GTP	Guanosine triphosphate
HBSS	Hank's buffered saline solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HDL	High density lipoproteins
H₂O₂	Hydrogen peroxide
HR	Hour

HRS	Hours
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium
I. P.	Intraperitoneal
IP₃	Inositol trisphosphate
KATP	Potassium ATP
KCl	Potassium chloride
KH₂PO₄	Potassium dihydrogen orthophosphate
KRBB	Krebs Ringer Bicarbonate Buffer
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight
MG	Milligrams
MgSO₄.7H₂O	Magnesium sulphate heptahydrate
MIN	Minute
MINS	Minutes
NaCl	Sodium chloride
NaHCO₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NIH	National Institute of Health
NiSO₄	Nickel sulphate
NPY	Neuropeptide Y
NSB	Non-specific binding
OEA	Oleoylethanolamide
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PEA	Palmitoylethanolamide
PFA	Paraformaldehyde
PKA	Protein kinase A

PKC	Protein kinase C
PP	Pancreatic polypeptide
PYY	Peptide YY
qPCR	Quantitative real-time PCR
RANTES	Regulated on activation, normal T cell expressed and secreted
RBP4	Retinol binding protein-4
RIA	Radioimmunoassay
RP-HPLC	Reverse-phase high performance liquid chromatography
SECS	Seconds
SGLT	Sodium glucose co-transport
STZ	Streptozotocin
SUR	Sulphonylurea receptor
TFA	Trifluoroacetic acid
TO	Traditional outbred
TRITC	Tetramethylrhodamine isothiocyanate
TZD	Thiazolidinediones
U	Units
WK	Week
WKS	Weeks
ZnCl₂	Zinc chloride

Declaration

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Abstracts

Miskelly, M. G., Flatt, P. R. and McKillop, A. M. 2016 Activation of GPR55 regulates glucose homeostasis and incretin secretion from intestinal L cells. *Irish Journal of Medical Science*. Irish Endocrine Society 40th annual meeting (Poster Presentation).

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Chapter 1

Introduction

1.1: Diabetes

Diabetes mellitus is a metabolic disorder which is defined by chronic hyperglycaemia which is caused by either defective insulin secretion, reduced sensitivity to insulin in peripheral tissues or both (Karamanou *et al.* 2015). In 2015, 415 million people were estimated to have diabetes with this number expected to rise to 642 million people in 2040 (Ogurtsova *et al.* 2017). A large number of people with diabetes have yet to be diagnosed (Shi, Hu 2014). Unchecked, hyperglycaemia leads to a range of complications including diabetic ketoacidosis and hyperosmolar hyperglycemic state (Kitabchi *et al.* 2009). Diabetes appears to be linked with increased cancer rates, however the mechanisms through which they are both linked are not yet understood (Shi, Hu 2014). Diabetes is also linked with cardiovascular diseases as well as complications in the eyes, kidneys and nerves due to damage to the small blood vessels (Emerging Risk Factors Collaboration *et al.* 2010).

1.1.1: Classification of diabetes

Diabetes was first classified into insulin-sensitive and insulin-insensitive types (Himsworth 2011). Up to 3 million people in the USA are estimated to be living with Type 1 Diabetes which is the insulin sensitive type described by Himsworth in 1936 (Chiang *et al.* 2014). It is estimated that around 190000 youths under 20 years of age have Type 1 Diabetes in the USA (Pettitt *et al.* 2014). In Type 1 Diabetes, lymphocytes target the β -cells of the pancreas leading to reduced insulin secretion (Craig, Hattersley & Donaghue 2009). Clinical symptoms of Type 1 Diabetes present themselves whenever T-cell mediated destruction of the β -cells has destroyed 90% of β -cell population in the pancreas (Gepts 1965). It can be hard to diagnose late onset Type 1 Diabetes, however 8.8% of people with this type of diabetes produce GAD antibodies (Hawa *et al.* 2013) while one in three people have residual C-peptide levels especially in those diagnosed later in life (Davis *et al.* 2015). Clinical symptoms of Type 1 Diabetes include polyuria, polydipsia, and ketonemia with the most dangerous side effect of these being the rapid onset of ketoacidosis (Chiang *et al.* 2014). Type 1 Diabetes accounts for approximately 5-10% of all diabetes cases (Mealey, Oates & American Academy of Periodontology 2006).

When Himsworth was describing the insulin-insensitive diabetes in 1936, he was most likely describing individuals with Type 2 Diabetes. This is the most prevalent form of diabetes with estimates suggesting 90% of all diabetes sufferers having Type 2 Diabetes (Zimmet, Alberti & Shaw 2001). The highest proportion of individuals with Type 2 Diabetes are those who have adopted a Western lifestyle while people from developing countries appear to be affected in lower numbers (Forouhi, Wareham 2014). Type 2 Diabetes is increasing in prevalence globally and there are expected to be approximately 600 million people diagnosed with Type 2 Diabetes by 2035 (Forouhi, Wareham 2014). The symptoms of Type 2 Diabetes include polyuria, polydipsia, polyphagia, and

weight loss (Vijan 2010). One of the main complications of Type 2 Diabetes is reduced glucose tolerance of the peripheral tissues due to reduced glucose uptake in these tissues during episodes of hyperinsulinemia and this complication can appear before the full development of Type 2 Diabetes (Lillioja *et al.* 1988, Warram *et al.* 1990, Lillioja *et al.* 1993). The most severe complications with Type 2 Diabetes are the hyperosmolar hyperglycemic state (HHS) and diabetic ketoacidosis, however diabetic ketoacidosis is rarely seen in people with Type 2 Diabetes (Pasquel, Umpierrez 2014, Fasanmade, Odeniyi & Ogbera 2008). Other complications seen in individuals with Type 2 Diabetes include altered lipid metabolism, retinopathy, nephropathy, neuropathy, impotence, acanthosis nigricans, or frequent infections (Vijan 2010, Nolan, Damm & Prentki 2011). These complications are strongly linked with obesity which has led to the development of the term “diabesity” (Shafir 1996). The therapies used to control the complications of Type 2 Diabetes include diet and drugs however if this doesn’t work then direct injection of insulin is required (Zimmet, Alberti & Shaw 2001).

The other main form of diabetes is gestational diabetes which occurs in pregnant women who are intolerant to glucose (American Diabetes Association 2004). It was originally described as a form of diabetes which increased foetal mortality however the same risk was not transferred to the mother (White 1949). Increased infant mortality is not the only risk for children as offspring from mothers affected by gestational diabetes are larger when born (Nolan 2011). Estimates suggest that 7% of all pregnant women are affected by gestational diabetes however estimates also vary from 1% to 14% of all pregnancies (American Diabetes Association 2004). Like Type 2 Diabetes, gestational diabetes is linked to obesity with heavier mothers being at greater risk of gestational diabetes (Chu *et al.* 2007a) however blood glucose is regulated better in women with reduced carbohydrate intake (Major *et al.* 1998). Gestational diabetes is also linked to an increased risk of developing Type 2 Diabetes in later life (Ross 2006).

1.1.2 Pathophysiology of type 2 diabetes

The development of Type 2 Diabetes has classically been attributed to 3 main causes of hyperglycaemia (Codario 2011). These are increased hepatic gluconeogenesis and glycogenolysis, impaired insulin secretion from the β -cells of the pancreas and resistance to insulin in the muscle and adipose cells of the body (Robert 1996). Despite several studies identifying loci which may confer an increased risk for Type 2 Diabetes (Hanis *et al.* 1996, Sladek *et al.* 2007), no single genetic cause has been identified (Codario 2011). On top of the 3 classical causes of hyperglycaemia in individuals with Type 2 Diabetes, a fourth cause, impaired incretin secretion, has been identified (Codario 2011). Evidence for insulin resistance was presented in 1960 in a study which demonstrated diabetic individuals having hyperinsulinemia as well as hyperglycaemia (Yalow & Berson 1960). Due to the complex nature of the pathophysiology of Type 2 Diabetes, there are

various treatments which are prescribed depending on the mechanisms causing hyperglycaemia or insulin resistance. These therapies are discussed in more detail below.

Increased gluconeogenesis has previously been described as the main cause of fasting hyperglycaemia (Consoli 1992). This is further backed as Metformin, the front line in Type 2 Diabetes treatment, exerts its mechanism of action through reduced gluconeogenesis (Hundal *et al.* 2000). Meanwhile, insulin resistance can occur naturally during different stages of the life cycle including puberty (Moran *et al.* 1999), aging (DeFronzo 1979) and pregnancy in women (Buchanan *et al.* 1990). The effects of insulin resistance have been shown to be reduced when physical activity is increased (Goodyear, Kahn 1998). The mechanism through which insulin resistance is increased appears to be through reduced GLUT4 expression and increased retinol binding protein-4 (RBP4) expression in adipocytes (Yang *et al.* 2005). Increased plasma free fatty acid levels have also been linked with insulin resistance (Boden 1997). Interestingly the function of the β -cells of the pancreas is mediated by the sensitivity of the peripheral tissues in the body to insulin (Kahn *et al.* 2006).

There is further evidence for the link between insulin sensitivity and the functionality of pancreatic β -cells as healthy β -cells respond to insulin resistance to maintain normal glycaemic levels, however when β -cells are dysfunctional, glucose homeostasis is impaired (Kahn *et al.* 2006). Dysfunctional β -cells lose function in various ways as they are unable to secrete insulin in response to both increased glucose levels and nonglucose secretagogues (Kahn 2001). Furthermore individuals with Type 2 Diabetes have exhibited reduced β -cell mass due to increased apoptosis resulting in the pancreas being unable to adequately respond to insulin secretory stimuli (Butler *et al.* 2003). The hyperglycaemia exhibited in Type 2 Diabetes may also play a role in β -cell dysfunction due to the toxic effects of elevated glucose levels on the β -cell (Garvey *et al.* 1985).

1.1.3: Current therapies

Before drug prescription for the treatment for Type 2 Diabetes, most people will try to use a form of dietary intervention to try and reduce chronic hyperglycaemia. Reduction in energy intake has been shown to normalise blood glucose values as well as reduce pancreatic fat (Lim *et al.* 2011). Undertaking a very-low-calorie diet (VLCD) of 600-700 calories a day can reverse Type 2 Diabetes in a very short timeframe however the long term effects of this diet are unknown (Steven *et al.* 2016). Other methods for weight loss include surgical intervention through bariatric surgery and this has shown to have positive effects on liver fat levels, insulin sensitivity, blood glucose levels, plasma insulin levels and body weight (Taylor 2008, Kashyap *et al.* 2010). These effects may be due to the area of the small intestine that is bypassed (Pories *et al.* 1987, Rubino *et al.* 2006). Bariatric surgery may be preferable to dieting as the long term weight loss through this method is sustainable (Pories *et al.* 1987) however this has to be measured against the potential for

complications during the surgery. When dietary intervention is unable to control the symptoms of Type 2 Diabetes, a pharmaceutical drug is prescribed and these are discussed in more detail below.

1.1.4: Biguanides

Biguanides were first used in medieval times through the use of the French lilac as an anti-diabetic therapy and became prominent in the 1960s (Witters 2001). The main biguanide used is metformin which is an oral drug which was approved by the FDA for the treatment of Type 2 Diabetes in 1995 (Hundal, Inzucchi 2003) however it was used in the UK from 1958 (Malek *et al.* 2013). Other biguanides such as phenformin and buformin had severe side effects which led to their withdrawal as an anti-diabetic treatment in the 1970s (Witters 2001). One of these side effects, lactic acidosis, is seen at far lower levels when metformin is administered (Sirtori *et al.* 1978) however it is more likely to occur in people with poor renal function (Lipska, Bailey & Inzucchi 2011). Metformin increases the insulin sensitivity of the liver and peripheral tissues as well as reducing hepatic glucose output (Dunn 2005, Hundal *et al.* 2000). The reduction of gluconeogenesis from the liver is insulin dependent and is regulated through the AMPK pathway (Malek *et al.* 2013). Metformin monotherapy is usually the first treatment adopted when a person is diagnosed with Type 2 Diabetes although combination therapies with insulin and other drugs are being investigated (Hemmingsen *et al.* 2012, Maruthur *et al.* 2016).

1.1.5: Sulphonylureas

Sulphonylureas have a direct effect on the insulin secreting β -cells of the pancreas (Rendell 2004). As a result of this one of the main complications with the use of sulphonylureas is hypoglycaemia (van Dalem *et al.* 2016). There are 3 forms of sulphonylurea receptor, SUR1, SUR2A and SUR2B (Matsuo *et al.* 2000) with the SUR1 receptor being expressed in the pancreas (Donley *et al.* 2005). Sulphonylureas bind to SUR1 which is a crucial component of ATP-sensitive potassium ion channels along with Kir6.2 subunits (Reis, Velho 2002). Four Kir6.2 subunits form the inner pore of the K_{ATP} channel through which potassium ions can enter the cell with each subunit being attached to a separate SUR subunit which ATP binds to (Nichols 2006). ATP binding to SUR1 is dependent on magnesium ions (Matsuo *et al.* 2000). These K_{ATP} channels are inwardly rectifying potassium channels which allow potassium to enter β -cells of the pancreas (Hibino *et al.* 2010). Sulphonylureas attach to these receptors, forcing them to close and as a result potassium can no longer enter the β -cell. This leads to membrane depolarisation, the influx of extracellular calcium into the cell and thus insulin secretion (Gribble, Reimann 2003). Knockout of either the Kir6.2 or SUR1 subunits of the K_{ATP} channel results in neonatal hypoglycaemia (Miki *et al.* 1998, Seghers *et*

al. 2000). Single nucleotide polymorphisms in either the Kir6.2 or SUR1 subunits can lead to hyperinsulinemic hypoglycaemia in infants as well as an increased risk in Type 2 Diabetes which is in line with the effects observed in the *in vivo* knockout models described above (Reis, Velho 2002).

1.1.6: Meglitinides

Like sulphonylureas, meglitinides increase insulin secretion through direct action on the K_{ATP} channels of the β -cells of the pancreas (Blickle 2006). Repaglinide was the first meglitinide used as an anti-diabetic therapy followed closely by nateglinide (Landgraf 2000). Oral administration of meglitinides leads to a rapid increase in insulin secretion from the pancreas and reduces postprandial hyperglycaemia (Black *et al.* 2007). They also do not have long lasting effects on the pancreas as they are rapidly metabolised in the liver (Landgraf 2000). This makes it meglitinides a viable alternative to those who cannot use metformin due to renal impairment (Blickle 2006). Meglitinides have also been shown to reduce glycosylated HbA1c with repaglinide outperforming nateglinide (Black *et al.* 2007). Compared to sulphonylureas, meglitinide administration confers an increased risk of hypoglycaemia (Gerich *et al.* 2005) while there was increased weight gain compared to metformin administration (Black *et al.* 2007). Due to their rapid degradation in the liver, meglitinides need to be taken in more frequent dosages than other anti-diabetic medications (Inzucchi *et al.* 2012).

1.1.7: Thiazolidinediones (TZDs)

TZDs, which are also known as glitazones, are similar to metformin as they increase insulin sensitivity of fat, liver and muscle cells (Hauner 2002). Administration of pioglitazone was shown to have no detrimental effects on cardiovascular risk and it has been shown to reduce coronary atherosclerosis (Mannucci *et al.* 2008, Nissen *et al.* 2008). It is thought that the reason for these beneficial side effects is due to the activation of the peroxisome proliferator-activated receptor gamma (PPAR- γ) by TZDs which in turn alters the expression of a range of genes involved in metabolism (Hauner 2002). TZDs can be administered as a monotherapy or as a combination therapy with other Type 2 Diabetes therapeutic drugs (Rosenstock *et al.* 2007). Side effects of TZDs include weight gain, water retention, increased risk of myocardial infarction and there is also data to suggest that there is an increased risk of bladder cancer (Nathan *et al.* 2006, Nissen, Wolski 2010, Ferwana *et al.* 2013).

1.1.8: α -glucosidase inhibitors

α -glucosidase inhibitors prevent postprandial hyperglycaemia through the reduction in the rate of gastric emptying by reducing the rate of polysaccharide digestion (Nathan *et al.* 2006). As α -glucosidase inhibitors work in the intestine, they have no direct effect on insulin secretion from the pancreas and thus are unlikely to cause hypoglycaemia (Van de Laar *et al.* 2005). They have been shown to exhibit positive effects on glycated HbA1c, fasting blood glucose and postprandial blood glucose (Van de Laar *et al.* 2005). Acarbose has also been shown to have positive results in the reduction of myocardial infarction, hypertension and other cardiovascular events (Chiasson *et al.* 2003). Due to their effects on the intestine, the main detrimental side effects of these inhibitors are found in the gastrointestinal tract and include flatulence, diarrhea and stomach ache (Van de Laar *et al.* 2005).

1.1.9: GLP-1 mimetics

As discussed above, incretin hormones are important regulators of glucose homeostasis however they are rapidly degraded by DPP-IV. Also described above are the GLP-1 receptor agonists Exenatide and Liraglutide. GLP-1 receptor agonists are an attractive area of research in Type 2 Diabetes research due to the ability of GLP-1 to increase β -cell mass through the generation of new β -cells and the reduction of β -cell apoptosis (Lund, Knop & Vilsboll 2011). GLP-1 mimetics are preferred to native GLP-1 as they avoid the rapid cleavage that GLP-1 undergoes by DPP-IV. Exenatide (marketed as Byetta) is based off the peptide Exendin-4 which was first extracted from *Heloderma suspectum* venom and has 53% sequence homology with GLP-1 (Eng *et al.* 1992). Despite the differences in sequence between Exendin-4 and GLP-1, there does not appear to be any differences in the potency of each agonist for the GLP-1 receptor (Lund, Knop & Vilsboll 2011). Liraglutide (which is marketed as Victoza) has 97% sequence homology with GLP-1 but avoids DPP-IV degradation through the addition of a 16-carbon fatty acid chain at Lys26 and the substitution of a Lysine residue with an Arginine residue at position 34 of the polypeptide chain (Neumiller *et al.* 2010). Exenatide was the first GLP-1 mimetic to be used as a therapy for Type 2 Diabetes with FDA approval being granted in 2005 with Liraglutide being granted this in 2010 (Lund, Knop & Vilsboll 2011). The first generation of GLP1R agonists have to be injected either once or twice daily which has led to the development of longer lasting agonists which are injected once a week such as Bydureon, Lixisenatide, Albiglutide, Dulaglutide and Semaglutide (Lund, Knop & Vilsboll 2011, Lund, Knop & Vilsboll 2014). The side effects of GLP1R agonists mostly stem from the gastrointestinal tract and include diarrhea, nausea and vomiting while spells of dizziness have also been reported (Agerso *et al.* 2002, Neumiller *et al.* 2010). The anti-diabetic effects of GLP-1 mimetics include reduced glycated HbA1c, fasting glucose and body weight (Buse

et al. 2004, DeFronzo *et al.* 2005, Kendall *et al.* 2005, Feinglos *et al.* 2005). GLP-1 mimetics may also have beneficial effects on fatty liver disease and inflammation (Lee *et al.* 2012, Samson, Bajaj 2013, Wang *et al.* 2014).

1.1.10: DPP-IV inhibitors

DPP-IV inhibitors improve glycaemic control through a reduction in the degradation of native GLP-1 (Pathak, Bridgeman 2010). The quick half-life of native GLP-1 in the body directed researchers towards the targeting of DPP-IV (Verspohl 2009). The first approved DPP-IV inhibitor was Sitagliptin which was approved by the FDA in 2006 (Dicker 2011). The effects of these compounds appears to be only through the incretin hormones and not a direct action on the pancreas as the use of DPP-IV inhibitors in double incretin receptor knockout (DIRKO) mice had no effect on glucose homeostasis (Hansotia *et al.* 2004). In high fat fed rats, DPP-IV inhibition reduced hyperglycaemia and increased insulin secretion through the incretin pathway after being challenged with glucose (Mitani *et al.* 2002). DPP-IV inhibitors have also shown the ability to reduce glycated HbA1c, fasting blood glucose, postprandial blood glucose, mean blood glucose and postprandial glucose levels as well as increase mean and postprandial GLP-1 levels (Ahren *et al.* 2004b, Dicker 2011). They can also be used as a monotherapy but are also used as a combination therapy with other treatments (Ahren *et al.* 2004a, Dicker 2011). Unlike GLP-1 mimetics, DPP-IV inhibitors can be administered orally (Verspohl 2009). There are a range of adverse side effects caused by DPP-IV inhibitors which include headaches, respiratory tract infection, urinary tract infection and, like metformin, should not be administered to people with renal impairment (Pathak, Bridgeman 2010).

1.1.11: Sodium-glucose cotransporter 2 (SGLT2) inhibitors

SGLT2 inhibitors are a relatively recent discovery for the treatment of Type 2 Diabetes and they have been recommended as a second option for individuals who do not respond or cannot use Metformin (Cefalu, Riddle 2015). SGLT2 inhibitors tend to be used as a double or triple combination therapy in conjunction with other diabetic medicines (Rosenstock *et al.* 2007, Lewin *et al.* 2015) and they have potential as a combination therapy with Metformin as SGLT2 inhibitors convey a protective effect on the kidneys (Fioretto *et al.* 2016). SGLT2 inhibitors were targeted as a therapy for Type 2 Diabetes as blood glucose is reabsorbed in the kidneys and inhibition of this reabsorption should lead to reduced blood glucose and better control of hyperglycaemia (Han *et al.* 2008). They have proven to be useful in the treatment of Type 2 Diabetes through the reduction of glycated HbA1c, body weight and systolic blood pressure (Leiter *et al.* 2015, Matthaei *et al.* 2015). The first SGLT2 inhibitor identified was dapagliflozin which reduced blood glucose in

hyperglycaemic rats (Meng *et al.* 2008). Other SGLT2 inhibitors have been identified and some of these have been shown to reduce blood pressure and hypertension in individuals with Type 2 Diabetes (Tikkanen *et al.* 2015). Care must be taken when administering SGLT2 inhibitors as they may cause diabetic ketoacidosis despite no dramatic increase in blood glucose (Rosenstock, Ferrannini 2015, Taylor, Blau & Rother 2015).

1.1.12: Amylin analogues

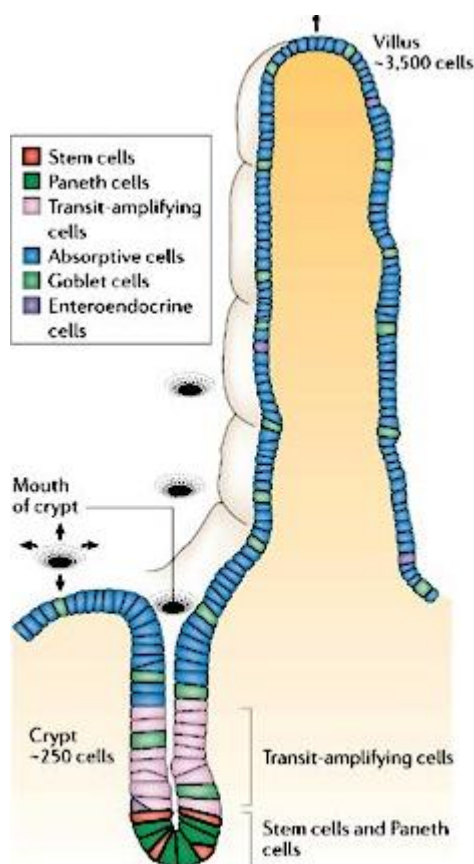
There is only one amylin analogue that is currently approved for use in the treatment of both Type 1 and Type 2 Diabetes and this is known as pramlintide (Jones 2007). There are 3 different substitutions which amylin undergoes in order to form pramlintide. The alanine at position 25 and serine residues at positions 28 and 29 in amylin are substituted for a proline residue in each position (Edelman, Maier & Wilhelm 2008). One of the major issues with medication for Type 2 Diabetes is the resulting weight gain, however pramlintide has shown that it can reduce the body weight as well as glycated HbA1c in obese individuals (Hollander *et al.* 2004). One of the issues with pramlintide is that it has to be administered via subcutaneous injection and as a result it is normally administered alongside insulin (Hollander *et al.* 2004, Ryan, Jobe & Martin 2005, Edelman, Maier & Wilhelm 2008). Further complications include nausea and hypoglycaemia (Ryan, Jobe & Martin 2005). It may also not be appropriate for those with poor renal function as pramlintide is broken down in the kidneys (Edelman, Maier & Wilhelm 2008).

1.2: Small intestine

The small intestine runs from the stomach to the caecum and is over 20ft long with the length of the intestine being correlated with the individual person's height (Underhill 1955). The surface area of the small intestine has been described to be a similar size to that of a tennis court (260-300m²) (Niess, Reinecker 2006) however more recent measurements describe a surface area that is 10 times less than this figure (30m²) (Helander, Fändriks 2014). The small intestine has numerous villi which project into the lumen of the gut through their finger-like shape (Clevers 2013). Villi are lined mainly with epithelial cells that provide a barrier to infection while also being closely linked to the vascular system which allows for the absorption of nutrients (Clevers 2013). At the bottom of the villi are the crypts of Lieberkühn where populations of intestinal stem-cells reside. These stem cells travel up the epithelial lining of the villi thus providing a self-renewal function for intestinal villi (Clevers 2013). Stem cells can be identified by the intestinal stem cell marker Lgr5 (van, Clevers 2009). The first part of the intestine, the duodenum, was first described by Herophilus of chalcidon

who was one of the first people to carry out autopsies on humans (Bay, Bay 2010). It is named as such due to the fact that it was found to be 12 finger widths long (Helander, Fändriks 2014). The duodenum is distinct from the jejunum and ileum due to the presence of Brunner's glands (Krause 2000). The jejunum is the middle part of the intestine and is $\frac{2}{3}$ the length of the ileum (Cornes 1965). The ileum is distinct from the other sections of the intestine due to the presence of Peyer's patches, however these are sometimes observed in the distal duodenum and the distal jejunum (Cornes 1965). The function of Peyer's patches is important for the role of the small intestine as a barrier to infection as the main function of these patches is mucosal immunity (Lelouard *et al.* 2012). Recently a new organ which interacts with the small intestine, known as the mesentery, has been described however further studies will need to be carried out to determine what role this organ plays in small intestinal function (Coffey, O'Leary 2016). The composition of the lining of the villi is not as simple as stem cells in the crypts of Lieberkühn while epithelial cells line the walls of the villi. Instead the nutrient absorbing epithelial cells are interspersed with enteroendocrine, goblet, Paneth, tuft and M cells (Clevers 2013) all of which arise from stem cells in the crypts of Lieberkühn (Gordon 1993) and all of which are described in more detail below (Figure 1.1).

Figure 1.1



Adapted from Crosnier, Stamataki & Lewis 2006. Paneth cells (dark green) which secrete antimicrobial peptides reside at the bottom of the intestinal crypt. Stem cells (red) are also found in the crypt and these cells can differentiate into any of the cell types found in the villi. This differentiation occurs in the region labelled the transit-amplifying cells (pink) which is just above the bottom of the crypt. After the crypt, the villi protrude into the lumen of the small intestine in a finger like conformation. Cells on the outer surface are determined to have an open conformation and these are mainly epithelial cells (in blue) which allow for the absorption of nutrients from digested food travelling through the intestine. Interspersed throughout the epithelial cells are goblet cells (light green) which secrete mucus to help with the transport of food along the lumen. Also interspersed throughout the epithelial cells are the hormone secreting enteroendocrine cells.

1.2.1: Enteroendocrine Cells

The gastrointestinal tract secretes a wide variety of hormones with over 30 different gastrointestinal hormones having been described (Gunawardene, Corfe & Staton 2011). Despite the large number of hormones secreted from the intestine, the enteroendocrine cells of the intestine only make up a small (less than 1%) proportion of the intestinal cell population (Sternini, Anselmi & Rozengurt 2008, Gunawardene, Corfe & Staton 2011) and are scattered individually throughout the intestine (Schonhoff, Giel-Moloney & Leiter 2004). The enteroendocrine cells can be further divided into G, X/A-like, D, I, K, L and enterochromaffin cells (Sternini, Anselmi & Rozengurt 2008) which are described in more detail below. Cells can be described as 'open' or 'closed' which describes their position on the villi. Open cells protrude into the lumen while closed cells do not (Sternini, Anselmi & Rozengurt 2008).

1.2.2: G cells

The G cells of the intestine secrete gastrin and are found in the upper duodenum as well as the stomach (Walsh, Grossman 1975). Secretion from G cells is potently stimulated by individual amino acids, phenylalanine and tryptophan in particular (Taylor *et al.* 1982) as well as extracellular calcium ions (Levant, Walsh & Isenberg 1973). Interestingly secretion from G cells is not influenced by the concentration of calcium ions in plasma. The reason for this is that extracellular calcium activates a calcium sensing receptor which in turn activates phospholipase C. This opens other cation receptors on the cell membrane thus allowing for the influx of calcium into the cell and thus secretion from intestinal G cells (Buchan *et al.* 2001). G cells are located on villi so project into the lumen and as a result are affected by the entry of nutrients into the lumen (Dockray, Varro & Dimaline 1996).

1.2.3: X/A-like cells

The naming of X/A-like cells has arisen due to their similarity to α -cells of the pancreas (thus the A-like name) and due to the inability to determine their function in rats (X cells) (Stengel, Tache 2009). Ghrelin, a hormone which is secreted in appetite regulation, was localised to the X/A-like cells in the gastrointestinal tract as well as the stomach thus providing some light on the function of these cells (Date *et al.* 2000). It was later elucidated that these cell also contain obestatin, a hormone which reduces appetite and was originally thought to be an agonist for GPR39 (Zhang *et al.* 2005a).

These cells are mainly located in the upper part of the gastrointestinal tract (Rindi *et al.* 2004) and are described as closed due to their unavailability to the lumen (Stengel, Tache 2009).

1.2.4: D cells

D cells secrete somatostatin and are found throughout both the small and large intestine (Sjolund *et al.* 1983). They appear less often than other enteroendocrine cell types as they make up approximately 3-5% of all enteroendocrine cells (Gunawardene, Corfe & Staton 2011). They are mainly found in the pancreas as discussed below.

1.2.5: I cells

I cells secrete CCK in response to fatty acids and proteins as they travel along the villi of the small intestine (Sternini, Anselmi & Rozengurt 2008). They are situated mainly in the duodenum and jejunum (Figure 1.2) and have the open conformation on the villi wall that allows them to interact with the contents of the lumen (Liddle 1997). There has been previous little research on I cells alone due to the difficulty in isolating these cells (Sykaras *et al.* 2012b). The lumen sensing mechanism of I cells has yet to be fully elucidated, however this is proposed to be through G-protein coupled receptors (GPCRs) and GPR40 has been identified on I cells in the intestine (Liou *et al.* 2011). This same research used GPR40 knockout mice to show that the phospholipase C pathway was the likely signalling mechanism for these cells. Another G-protein coupled receptor through which I cells may be activated is GPR120 however the role of this receptor in CCK secretion has yet to be fully understood (Sykaras *et al.* 2012b). There is also evidence that GPR41 and GPR43 may be other potential G-protein coupled receptor on I cells (Samuel *et al.* 2008, Sykaras *et al.* 2012b)

1.2.6: K cells

Originally K cells were thought to be functional stages of other enteroendocrine cells, however in 1975 they were discovered to be the GIP secreting cell of the intestine which are present in both the duodenum and jejunum (Buffa *et al.* 1975). While K cells are considered to be a distinct cell type from other enteroendocrine cells, there is a small proportion of cells in the intestine which secrete both GIP and GLP-1 which is usually secreted from L cells (Mortensen *et al.* 2003). GIP secretion from K cells appears to be controlled through 2 separate pathways, with the nutrient sensing

pathway leads to AMPK-kinase regulation while the neuronal pathway activates protein kinase C (Li, Wice 2005). The receptors present on the membrane of the K cell appear to provide clues to which nutrients stimulate GIP secretion with both glucose sensing and fatty acid sensing receptors being present (Parker *et al.* 2008). K cells are of interest in diabetes research not only for their GIP secreting effects, with recent studies engineering them to produce insulin as a possible therapy (Mojibian *et al.* 2014)

1.2.7: L cells

Intestinal L cells secrete both GLP-1, PYY and GLP-2 and are more numerous in the ileal portion of the small intestine than the duodenal section (Sternini, Anselmi & Rozengurt 2008, Habib *et al.* 2012, Petersen *et al.* 2014). L cells are normally distinguished from other enteroendocrine cells due to the expression of the preproglucagon gene however there is research showing that the location of the cell is of more importance than the genes produced (Habib *et al.* 2012). The presence of glucose can affect L cell secretion due to the depolarisation of the membrane leading to increased intracellular calcium (Reimann *et al.* 2008) with the knockout of the GLUT2 receptor leading to reduced GLP-1 secretion (Cani *et al.* 2007). The presence of fatty acid receptors on the L cell membrane provide evidence that fatty acids also regulate L cell secretion (Reimann *et al.* 2008) while L cell secretion can also be regulated through the cAMP pathway (Tolhurst *et al.* 2011a).

1.2.8 Enteroendocrine cell plasticity

The enteroendocrine cells of the intestine have classically been categorised into cell types based on their function and hormonal secretion as described above. Recent advances have suggested that segregation of enteroendocrine cells into distinct cell sub types does not accurately describe the nature of enteroendocrine cells. Differentiation of stem cells in the intestinal crypt into endocrine cells of the epithelium is regulated by a number of transcriptional factors such as Math1, Neuro3, NeuroD and Hes1 (May, Kaestner 2009, Li *et al.* 2011). Enteroendocrine cell differentiation is also regulated by various transcription factors along the cephalocaudal axis of the intestine with factors such as PDX-1, CDX-2, GATA-4, GATA-5, GATA-6, HNF-1 α , HNF-1 β and CDP all showing distinct expression patterns in different areas of the intestine (Fang, Olds & Sibley 2006, Middendorp *et al.* 2014). A number of studies have identified co-expression of hormones in enteroendocrine cells of the intestine outside of enteroendocrine cell classifications based on hormonal secretion (Habib *et al.* 2012, Egerod *et al.* 2012, Svendsen *et al.* 2015, Cho *et al.* 2015).

Knockout models have demonstrated that the absence of these transcription factors can lead to changes in the population of enteroendocrine cells in the villi (Larsson *et al.* 1996, Jepeal, Boylan & Wolfe 2003, Jepeal *et al.* 2005). Manipulation of these factors may have potential in changing the enteroendocrine cell population of the intestine (Gribble, Reimann 2016).

1.2.9: Enterochromaffin cells

Enterochromaffin cells are neuroendocrine cells which make up less than 1% of the intestine (Bellono *et al.* 2017). These cells secrete serotonin which was described in 1948 (Rapport, Green & Page 1948) and produce histamine (Hakanson *et al.* 1986, Delwaide *et al.* 1991). One of the main characteristics of enterochromaffin cells is the electron dense core of the secretory granules present within the cell (Hakanson *et al.* 1992). These cells are also sensitive to circulating gastrin levels, with high levels of gastrin resulting in the secretion of histamine while high levels of circulating somatostatin may have a paracrine effect on these cells (Hakanson *et al.* 1992). Long term exposure to high levels of gastrin leads to enterochromaffin cell hypertrophy (Bottcher *et al.* 1989) and hyperplasia if this effect is sustained (Hakanson *et al.* 1992). The histamine secretory role of enterochromaffin cells is of particular importance as long term inhibition of histamine secretion resulted in inhibition of gastric acid secretion and cancerous tumours in the enterochromaffin cells of the stomach (Havu 1986). The mechanism that gastrin uses to increase histamine secretion appears to be through binding to a membrane receptor which releases calcium into the cytoplasm and also depolarises the membrane to increase intracellular calcium (Prinz *et al.* 1993). This same study also demonstrated an effect of CCK-8 on these cells. This is not the only mechanism through which histamine is released as further research has shown that enterochromaffin cells are able to receive signals from the nervous system (Bellono *et al.* 2017).

1.2.10: Goblet cells

The main role of goblet cells in the intestine is to secrete mucus into the lumen of the intestine which provides lubrication and protection to the villi (Forstner 1978). They have been named goblet cells due to their cup-like shape which occurs when the supranuclear portion of the cell fills with mucus (Birchenough *et al.* 2015). Unlike the stomach and large intestine, the small intestine has one mucus layer which protects the cells of the villi (Ermund *et al.* 2013). Goblet cells arise from 2 main precursor cells in the small intestine. One precursor cell is the oligomucous cell which are cells which contain small amounts of mucus but can become mature goblet cells as they migrate up the

villi (Cheng 1974). Oligomucous cells are present in the base of the crypts of Lieberkühn and the lower half of the midcrypt compartment while mature goblet cells appeared higher in the crypt and also appeared in the villi (Cheng 1974). Oligomucous cells account for around half of all mucous containing cells in the intestine. Therefore there must be another pathway for the development of mature goblet cells. The other half of the goblet cell population appears to arise from columnar stem cells which exist at the bottom of the crypts (Cheng, Leblond 1974). Further evidence for goblet cell development occurring through two pathways is that goblet cells travel up the villi and eventually disperse into the lumen once at the tip and the oligomucous cell pathway is unable to keep up with the rate of goblet cell turnover (Merzel, Leblond 1969). The amount of mucus stored in goblet cells increases from the duodenum until around midway through the jejunum, after which mucus levels stay relatively constant (Kemper, Specian 1991). One source for mucus secreted from goblet cells is glucose and the mucus they secrete is produced at a constant rate (Neutra, Leblond 1966). There are approximately 50 proteins which make up the proteome of intestinal mucus with the major component MUC2 being the best understood (Birchenough *et al.* 2015). One of the main stimulators of mucus secretion from goblet cells is acetylcholine (Specian, Neutra 1980) while secretion is also stimulated by histamine (Halm, Halm 2000).

1.2.11: Paneth cells

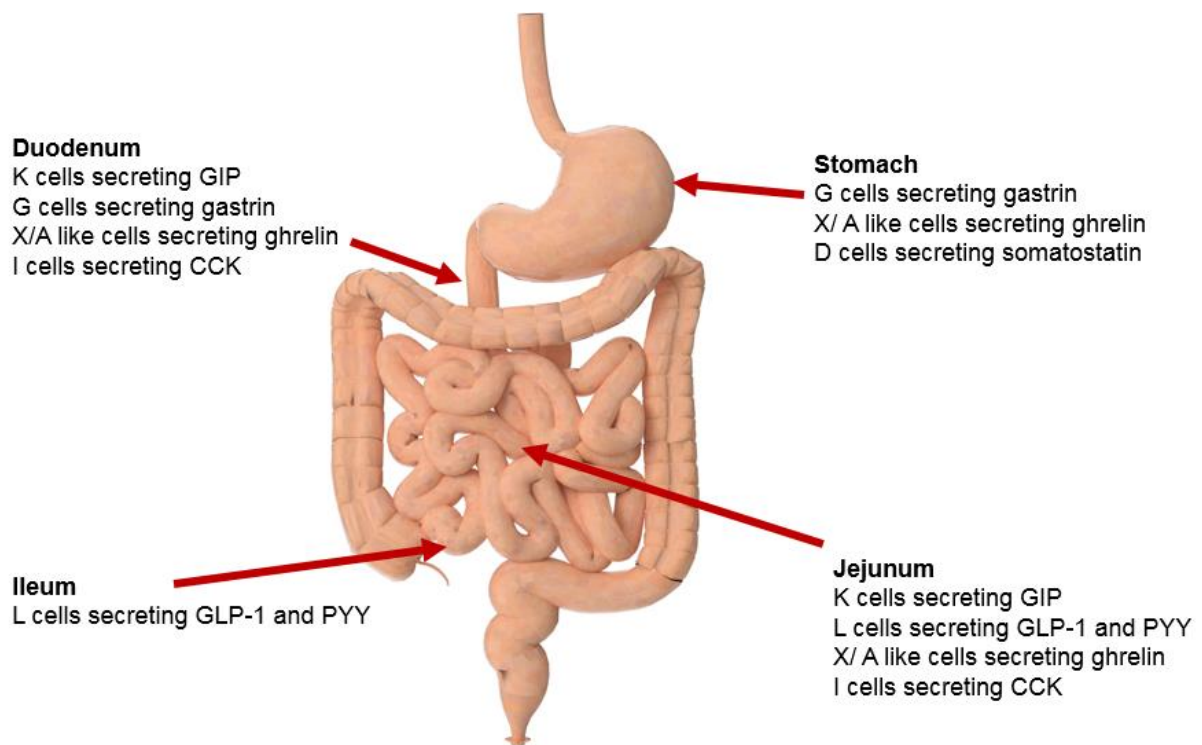
As described above, goblet cells provide a protective mucus layer which prevents direct contact between cells of the villi and bacteria which live in the gut. However, in diseases such as inflammatory bowel disease this defence is broken down and bacteria can have a detrimental effect on the epithelial lining of the small intestine (Xavier, Podolsky 2007). There is a second defence mechanism in the intestine, which lies at the bottom of the crypts of Lieberkühn, and these are bacterial sensing cells that secrete antimicrobial peptides known as Paneth cells (Ayabe *et al.* 2000). The majority of the secreted peptides are alpha-defensins which were shown to protect the intestine from a range of pathogens (Ayabe *et al.* 2000) while angiogenin, lysozyme and intestinal phospholipase A2 proteins are also secreted (Sato *et al.* 1988, Harwig *et al.* 1995, Hooper *et al.* 2003). The mechanism through which Paneth cells recognise bacterial pathogens is the MyD88-dependent toll-like receptor (TLR) which activated through direct contact with bacteria (Vaishnava *et al.* 2008). The position of Paneth cells in the crypts of Lieberkühn suggests that their main defence role is the protection of intestinal stem cells which are also present at the bottom of the crypt (Ganz 1999). This could also be defined as a self-protective role as Paneth cells arise from the stem cells which they protect (Ganz 2000, Cheng, Leblond 1974). Another defining feature of Paneth cells is

that the epithelial cells of the small intestine have a high turnover rate, whereas Paneth cells are able to survive up to 30 days (Clevers, Bevins 2013).

1.2.12: Tuft cells

Tuft cells were first visualised in rat duodenum in 1973 (Isomaki 1973). One of the defining characteristics of tuft cells is the presence of numerous microvilli which extend into the lumen of the intestine from the apical surface of the membrane (Hoover *et al.* 2017). Like the cell types described above, the presence of the Lgr5 marker shows that these cells are derived from stem cells at the bottom of the crypts of Lieberkühn (Gerbe *et al.* 2011). Tuft cells can be distinguished from other intestinal cell types due to the DCLK1 expression (Gerbe *et al.* 2011). Despite the identification of intestinal tuft cells in 1973, only recently have there been studies carried out to determine their function. This may be due to the difficulty in identifying the different cells in the epithelial layer of the villi of the small intestine (Gerbe *et al.* 2009). One function which has been suggested is the secretion of opiod proteins (Kokrashvili *et al.* 2009). While the turnover of tuft cells in the villi is normally less than 2 weeks, there is a subpopulation that are long lived and are oncogenic when induced by inflammation (Westphalen *et al.* 2013). Another outstanding feature of tuft cells is their interaction with the nuclei of neighbouring cells using cryospinules (Hoover *et al.* 2017) and there is an absence of secretory granules within the cytoplasm of these cells (Morrone, Cangiotti & Cinti 2007). A second function for tuft cells that has been suggested is as the chemoreceptor cells of the intestine due to the presence of α -gustducin which is a component of the taste receptor in the tongue (Hofer, Puschel & Drenckhahn 1996).

Figure 1.2



The enteroendocrine cells are spread throughout the gastrointestinal tract. K cells, G cells, I cells and X/ A like cells are spread throughout the upper and middle of the small intestine and are also found in the stomach. Meanwhile L cells are found further down the small intestine and are mainly situated in the ileum.

1.3: Gastrointestinal hormones

1.3.1: Gastric inhibitory peptide/ Glucose-dependent insulintropic peptide (GIP)

GIP is secreted from the nutrient sensing K cells of the small intestine primarily in the duodenum in response to digestion of fatty acids and carbohydrates (Cleator, Gourlay 1975). GIP is a 42 amino acid polypeptide which is derived from a 153 amino acid long polypeptide precursor known as preproGIP (Takeda *et al.* 1987). GIP was first isolated in 1969 with further isolation in 1970 confirming the ability of GIP to inhibit gastric secretion (Brown *et al.* 1969, Brown, Mutt & Pederson 1970). This activity is why GIP received one of its monikers. Initially a 43 amino acid sequence for GIP was elucidated in 1971 (Brown, Dryburgh 1971) however this was corrected and after the removal of a glutamine residue at position 29, the currently accepted 42 amino acid

conformation was derived (Jörnvall *et al.* 1981). As the alternative name for GIP suggests, it is a hormone with insulintropic activity. This was first described in 1973 which showed that GIP administration to healthy humans resulted in increased insulin secretion (Dupre *et al.* 1973). Further research into this effect showed that GIP secretion is glucose sensitive and relies on hyperglycaemic conditions regardless of the level of insulin secretion (Andersen *et al.* 1978). The removal of GIP from rat gut extracts which were then administered to rats resulted in maintenance of more than 50% of the incretin effect (Ebert, Unger & Creutzfeldt 1983). This research suggested that GIP was not the only incretin hormone which led to the later discovery of glucagon-like peptide 1 (GLP-1). The actions of GIP are regulated in the body through a proteolytic enzyme dipeptidyl peptidase IV (DPP-IV) which cleaves GIP at the alanine residue in position 2 of the polypeptide chain, resulting in the inactive GIP (3-42) (Figure 1.4) and a short half-life of around 7 minutes (Kieffer, McIntosh & Pederson 1995).

The insulintropic effect of GIP makes GIP-based therapies, such as GIP mimetics, an attractive prospect in Type 2 Diabetes therapeutics. However, research in individuals with Type 2 Diabetes has shown that GIP loses its insulintropic action and this is one reason GIP mimetics are not more extensively studied (Nauck *et al.* 1993, Seino, Fukushima & Yabe 2010). This research discovered that after oral glucose administration, GIP secretory levels were not different between diabetic and non-diabetic subjects. This was the first work to demonstrate a decreased incretin effect in people affected by Type 2 Diabetes.

1.3.2: Glucagon-like peptide 1 (GLP-1)

GLP-1 is secreted from intestinal L cells in response to fatty acid and carbohydrate digestion (Hansen *et al.* 2013). Intestinal L cells are dispersed throughout the intestine however the highest concentration can be found in the distal ileum in the small intestine while the colon has lower concentrations than those found in the rectum (Adrian *et al.* 1985). The gene which codes for the GLP-1 protein, the proglucagon gene, codes for a precursor protein from which a number of peptides can be cleaved (Jin 2008). One interesting feature of the GLP-1 precursor protein is that the main hormone cleaved in the intestine, GLP-1, has the opposite function to that of the main hormone cleaved in the pancreas, glucagon. In order for these different hormones to be cleaved, they must undergo different post translational processing. In the intestine, post translational processing is regulated by the prohormone convertase-1 (PC-1) enzyme (Drucker, Nauck 2006). In the pancreas this processing is carried out by PC2 as described above (Rouille *et al.* 1994). The proglucagon gene is expressed throughout the body with mRNA transcripts being expressed in the brainstem, pancreas, and intestine, however post translational processing is tissue specific (Lee, Brubaker & Drucker 1990). Three main forms of GLP-1 circulate in the body – the full length 37

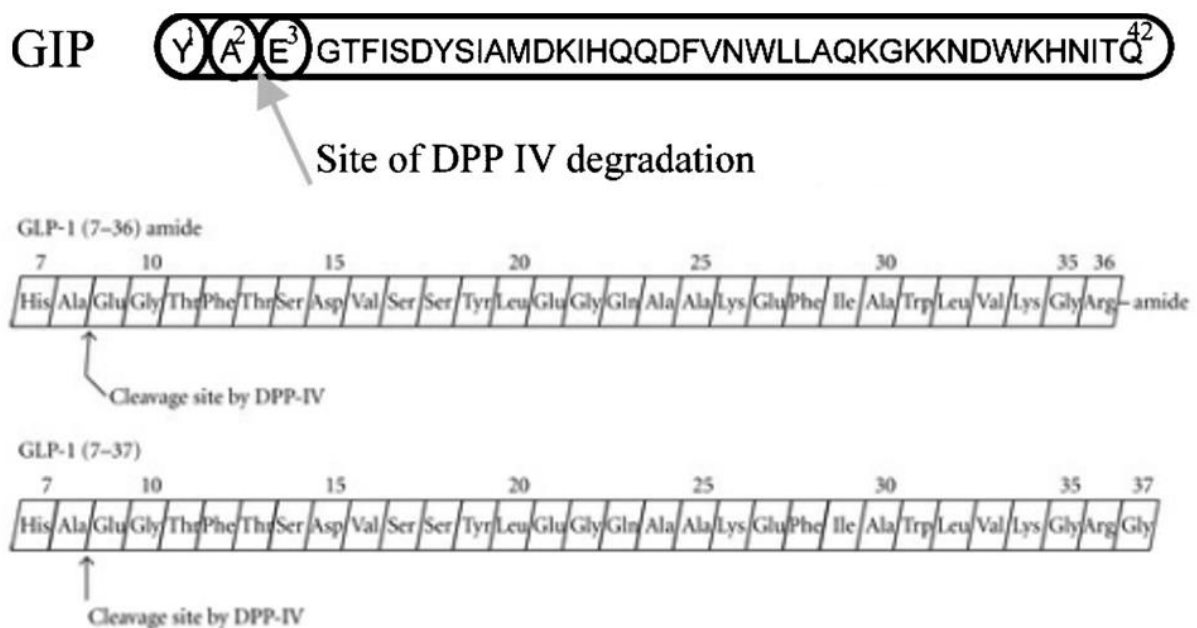
amino acid transcript known as GLP-1(1-37), the 31 amino acid long GLP-1(7-37) and the 30 amino acid GLP-1(7-36). All three forms of the peptide have been shown to increase insulin secretion (Drucker *et al.* 1987, Nauck *et al.* 1993, Suzuki, Nakauchi & Taniguchi 2003) while the GLP-1(7-37) form of GLP-1 increasing insulin gene expression in rats (Drucker *et al.* 1987) and GLP-1(7-36) has been suggested as a neuropeptide (Blazquez *et al.* 1998). GLP-1 was first described as an incretin in 1987 in non-diabetic human volunteers (Kreymann *et al.* 1987) where administration of GLP-1(7-36) increased plasma insulin as well as decreased blood glucose and glucagon. The insulinotropic effect of GLP-1(7-36) was determined to be greater than the other recognised incretin, GIP.

As described above, GIP loses its insulinotropic effects in individuals with Type 2 Diabetes despite no changes in GIP secretion levels. However, both GLP-1(7-36) and GLP-1(7-37) maintain their insulinotropic ability in individuals with Type 2 Diabetes. This was confirmed through studies between people with Type 2 Diabetes and people who were able to regulate their glucose homeostasis (Nathan *et al.* 1992, Nauck *et al.* 1993). This confirmed the previous effects seen in healthy human volunteers (Drucker *et al.* 1987, Nauck *et al.* 1993, Suzuki, Nakauchi & Taniguchi 2003). The insulinotropic effect of GLP-1 in individuals with Type 2 Diabetes make it an interesting therapeutic target. In fact GLP-1 mimetics have been adopted as clinical therapies since 2005 (Ahren 2009) with DPP-IV inhibitors being approved shortly thereafter in 2006 (Dicker 2011). GLP-1 has a short half-life due to its rapid cleavage by DPP-IV into the inactive GLP-1(7-36) and GLP-1(9-37) forms (Figure 1.4). In order to try and avoid the effects of DPP-IV, compounds which mimic GLP-1 but have slightly different structures, GLP-1 mimetics, have been developed. The two main GLP-1 mimetics used as therapeutic treatments targeting Type 2 Diabetes are exenatide and liraglutide (Lee *et al.* 2014). In order to avoid the cleavage effects of DPP-IV, the second position of the polypeptide chain in exenatide is modified as a Glycine residue replaces an Alanine residue. For Liraglutide, the effects of DPP-IV cleavage is reduced through two modifications. Firstly in the 34th position of the polypeptide chain Arginine is replaced with Lysine. The second modification seen is at position 26 of the polypeptide chain, where a Glutamate residue connected to a 16 carbon fatty acid chain is added.

The continued insulinotropic effect of GLP-1 in individuals with Type 2 Diabetes is not the only attractive feature of GLP-1 based therapeutics. The receptor through which GLP-1 is active, the GLP-1 receptor (GLP1R), is expressed in both α - and β -cells whereas the receptor through which GIP is active, the GIP receptor (GIPr), is only found on β -cells (Drucker, Nauck 2006). As GLP-1 can reduce glucagon secretion through the α -cells, it may help reduce the hyperglycaemia seen in Type 2 Diabetes. Another complication in Type 2 Diabetes is a reduction in β -cell mass and GLP-1 has been shown to reduce β -cell apoptosis adding to the mounting evidence of GLP-1 based therapies being a useful tool against Type 2 Diabetes (Farilla *et al.* 2003). Furthermore, Type 2

Diabetes has strongly linked to obesity and GLP-1 slows gastric emptying and reduces appetite making GLP-1 mimetics which retain this ability desirable (Nauck, Meier 2005). Currently GLP-1 mimetics depend on blood glucose levels meaning GLP-1 mimetics will only work in hyperglycaemic conditions. Another issue is that currently available therapeutics need to be injected. Potential combination therapies of GLP-1 mimetics and DPP-IV inhibitors such as Vildagliptin and Sitagliptin could help to increase the effectiveness of these therapies (Ceriello *et al.* 2014).

Figure 1.3



Adapted from Flatt, Green 2006, Kim, Kim 2012. GIP and GLP-1 are cleaved by DPP-IV at alanine residues which are at position 2 of the active peptides.

1.3.3: Peptide YY (PYY)

PYY was first described in 1980 as a 36 amino acid peptide that was present in the brain and intestine of pigs (Tatemoto, Mutt 1980). PYY is present in the L-cells of the small intestine along with GLP-1 and it is more concentrated towards the distal end, with the highest concentrations being found in the ileum (Adrian *et al.* 1985). The full length peptide can bind to various Y receptor subtypes such as Y1, Y2 and Y5 (Batterham, Bloom 2003). Similarly to GLP-1, PYY is cleaved by DPP-IV to form the 34 amino acid long PYY(3-36) which activates the Y2 receptor (Medeiros, Turner 1994). Both PYY(1-36) and PYY(3-36) are the main forms of PYY which circulate around the body in the blood (Grandt *et al.* 1994). PYY is released in response to the intake of nutrients

(Adrian *et al.* 1985) with the maximal levels of PYY being seen 90 minutes postprandially (Batterham, Bloom 2003). Administration of PYY(3-36) has been shown to reduce appetite and weight gain as well as increase insulin sensitivity (Batterham *et al.* 2002, Vrang *et al.* 2006) and these effects on appetite appear to be through Y2 receptor activation in the brain (Batterham, Bloom 2003). PYY can also reduce gastric emptying (Savage *et al.* 1987) and the effects of PYY make it and the receptors it activates attractive targets in the treatment of metabolic disorders such as obesity and Type 2 Diabetes.

PYY is also present in the endocrine cells of the pancreas and as seen in the small intestine it is co-localised with the proglucagon protein (Ali-Rachedi *et al.* 1984) as well as being observed in the PP secreting cells (Bottcher *et al.* 1993). It appears that PYY activation of the Y1 receptor is essential for the storage of various intestinal and pancreatic hormones which interestingly include insulin despite PYY not being present in the β -cells of the pancreas (Sam *et al.* 2012). Further evidence for this includes the inhibition of glucose and carbachol stimulated insulin secretion by PYY(1-36) and PYY knockout leading to hyperinsulinaemia (Persaud, Bewick 2014). The shortened form of the protein, PYY(3-36), has been shown to have no effect on insulin secretion (Chandarana *et al.* 2013) despite showing the ability to inhibit insulin secretion *in vitro* (Khan *et al.* 2016). Both the full length and shortened forms of PYY increased β -cell proliferation and as a result increased pancreatic β -cell mass which is one of the major complications seen in diabetes (Khan *et al.* 2016).

1.3.4: Cholecystokinin (CCK)

CCK is present in both the duodenum and jejunum of the small intestine (Buffa, Solcia & Go 1976) where it is secreted from the I cells (Buchan *et al.* 1978). The gene that codes for the CCK precursor protein is located on chromosome 3 in humans (Lund *et al.* 1986) and codes for a 115 amino acid long protein (Takahashi *et al.* 1985). This protein is post translationally processed into various forms of CCK with the most abundant in humans being CCK-33 closely followed by CCK-22 (Rehfeld *et al.* 2001). CCK is released from the small intestine in response to a wide variety of stimuli including glucose, amino acids and fatty acids (Liddle *et al.* 1985). CCK receptors are found throughout the body with the CCK1 receptor being predominantly found in the gastrointestinal tract and CCK2 receptor being more predominant in the brain (Wank 1995). Therefore CCK has a variety of functions including gallbladder contraction, decreased appetite and amino acid directed insulin secretion (Liddle *et al.* 1985, Rushakoff *et al.* 1987a, Gibbs, Young & Smith 1997) all of which can be utilised in the treatment of metabolic disorders such as Type 2 Diabetes.

1.4: Pancreas

The pancreas is an organ found in the upper abdomen in humans. It is separated into different regions such as the head, neck, body and tail with the head of the pancreas being attached to the duodenum of the small intestine by the ampulla of Vater (Slack 1995). These regions of the pancreas are well defined in humans with the head being located at the proximal end and the tail located at the distal end of the pancreas, however in rodents this is less defined. The pancreas is made up of exocrine glands which are acinar in shape (Pictet *et al.* 1972) and secrete enzymes into the small intestine which help with the absorption of nutrients from food (Slack 1995) as well as endocrine glands. The endocrine glands are arranged into islets of Langerhans which were first described in 1869 (Langerhans, Paul, Friedrich-Wilhelms-Universität Berlin, Medizinische Fakultät, 1869). In humans, pancreatic islets consist of β , α , δ , ϵ and PP-cells which are scattered about the islet with no particular order (Brissova *et al.* 2005, Cabrera *et al.* 2006) and make up 1-2% of the pancreas (Pour, Standop & Batra 2002). This same conformation is seen in monkeys while in mice and pigs a more structured conformation is observed. These cells were found to be closely related to the vascular system in humans with 48 to 59% of the pancreas being made up of β -cells. 33 to 46% α -cells and 8 to 12% δ -cells (Cabrera *et al.* 2006). However, the section of the pancreas can affect the composition of the islet, with the islets found in the head of the pancreas being shown to be rich in PP-cells while the rest of the pancreas was rich in α -cells (Orci *et al.* 1978, Malaisse-Lagae *et al.* 1979, Stefan *et al.* 1982). This appears to be conserved in mammalian species as rat pancreas has been shown to have the same PP-rich regions in the uncinate process of the head of the pancreas (Elayat, el-Naggar & Tahir 1995). Despite the differences in the composition between human and rat islets, and despite the fact that a human pancreas is larger with many more islets than a rat pancreas, the overall islet size appears to be conserved between species which suggests that there is an optimal size at which they work (Henderson 1969). Pancreatic islets are also essential in the development of a pancreas as well as the duodenum of the small intestine during embryogenesis as the mutagenesis of insulin-promoter-factor 1 (also known as PDX1) has led to the absence of this organ completely (Jonsson *et al.* 1994, Offield *et al.* 1996).

1.4.1: Insulin

Insulin was first discovered in 1922 by both Frederick Banting and Charles Best who demonstrated extracts from dog pancreatic islets had a powerful anti-diabetic effect (Banting *et al.* 1922). The first clinical trials using insulin commenced in 1922 with Frederick Banting and John McLeod (Banting, Best & Macleod 1922), who provided the laboratory for the work to be undertaken and supervised the research, both receiving the Nobel Prize in Physiology or Medicine in 1923 for the discovery (Raju 1998). Following the discovery of insulin, monomeric insulin was isolated using

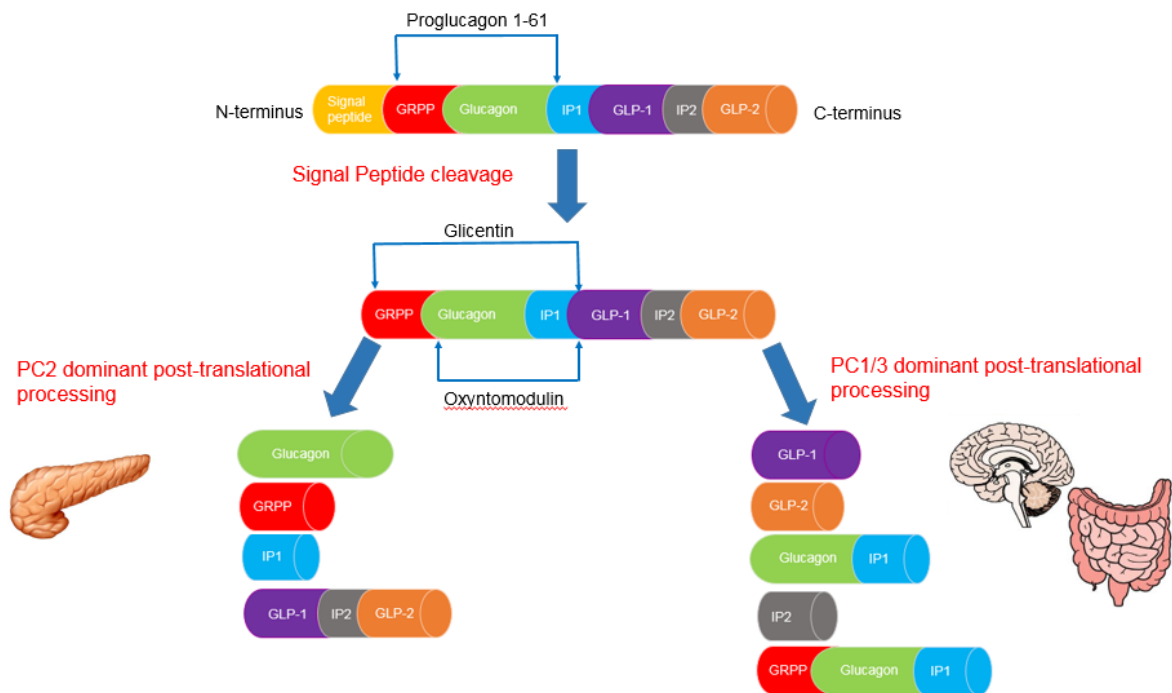
X-ray crystallography (Abel 1926) with the B chain of 30 amino acids being described in 1951 and the A chain of 21 amino acids being sequenced in 1953 (Sanger, Tuppy 1951a, Sanger, Tuppy 1951b, Sanger, Thompson 1953a, Sanger, Thompson 1953b) while the hexamer conformation in which insulin is stored being elucidated at a later date (Blundell *et al.* 1972, Crowfoot 1935). The sequencing work carried out by Frederick Sanger was the first time a protein sequence had been described. Despite this, due to difficulties in isolating the insulin receptor, the binding between insulin and the insulin-receptor was not visualised until 2013 (Menting *et al.* 2013). The gene coding the insulin protein is found on chromosome 11p15.5 (Owerbach *et al.* 1980, Owerbach *et al.* 1981). The main physiological role of insulin is the regulation of hepatic glucose output while it also facilitates the entry of glucose into peripheral tissues via the GLUT4 receptor (Sonksen, Sonksen 2000). The mechanism through which insulin regulates hepatic glucose production is via FoxO6 inhibition (Kim *et al.* 2011). Insulin has been proven to regulate free fatty acid release alongside hepatic glucose production in humans (Hall, Saunders & Sönksen 1979). It is stored in its inactive hexamer configuration (Sudmeier *et al.* 1981) in storage vesicles which are rich in zinc and calcium ions inside the β -cells of the pancreas (Dunn 2005). Insulin is released from these vesicles whenever the threshold potential of approximately -50mV is reached (Ashcroft, Rorsman 1989) and this potential is proportional to blood glucose levels with levels below 3mM leading to a resting potential around -70mV and no insulin secretion while concentrations of 7mM and above will induce the threshold potential. The main stimulus for insulin secretion is glucose which activates the respiratory chain (Soejima *et al.* 1996). The ATP molecules generated by the mitochondrial action causes depolarisation of the membrane due to the closure of ATP sensitive K_{ATP} channels and the opening of Ca^{2+} channels which allow calcium ions into the cell and thus leads to insulin exocytosis (Komatsu *et al.* 2013). The other main stimuli for insulin secretion include the incretin hormones, GLP-1 and GIP, short-term free fatty acid exposure (Komatsu, Sharp 1998) and parasympathetic nervous system activation (Ahren 2000).

1.4.2: Glucagon

Following on from the discovery of insulin in pancreatic extracts in 1922, glucagon was first described the following year in 1923 (Kimball, Murlin 1923) with the amino acid sequence of the 29 amino acid long peptide being elucidated in 1957 (Bromer, Sinn & Behrens 1957). A role for glucagon in glucose homeostasis was first described in 1969 when it was shown that glucagon secretion was blocked by induced hyperglycaemia in dogs (Ohneda *et al.* 1969). This discovery led to increased interest in the role of glucagon in diabetes and it was shown that glucagon levels were increased in those who underwent diabetic ketoacidosis (Gerich *et al.* 1975). The framework of the vasculature in the pancreas provides evidence of the role of glucagon, as capillaries are first

directed towards the β -cells of the pancreas before reaching the glucagon secreting α -cells (Taborsky 2010). These capillaries then run into the hepatic portal vein. Therefore this provides evidence that secretion from β -cells will affect the secretion from alpha cells and that the main effect of glucagon and insulin will be seen in the liver. Stimulation of the sympathetic nervous system leads to increased glucagon secretion (Marliss *et al.* 1973, Ahren, Veith & Taborsky 1987) which can be activated by hypoglycaemia (Havel, Munding & Taborsky 1996). Parasympathetic nerve stimulation was also shown to increase glucagon secretion, however the response to stimulation was less than that seen for insulin as well as sympathetic nerve effects on glucagon (Ahren, Taborsky 1986). The effect seen by parasympathetic nervous system activation appears to be reduced in diabetes, however this activity can recover through carbachol treatment (Patel 1984). The gene which codes for glucagon contains six exons and codes for a variety of proteins (Heinrich, Gros & Habener 1984). The tissue that the proglucagon gene is expressed in regulates which of these proteins are cleaved from the precursor protein through post translational processing (Mojsov *et al.* 1986). In α -cells the active glucagon protein along with a major proglucagon fragment consisting of GLP-1 and GLP-2 which are joined by IP2 are expressed while in the brain and small intestine active forms of GLP-1 and GLP-2 are released along with oxyntomodulin and glicentin, both of which include the whole glucagon molecule (Taborsky 2010). Differences in proglucagon gene processing between tissues can be explained through the enzyme which regulates the post translational modification of the precursor protein. In the pancreas this is carried out by PC2 (Rouille *et al.* 1994) while in the intestine it is regulated by PC1/3 (Tucker, Dhanvantari & Brubaker 1996). The main physiological effect of glucagon can be seen in the liver (Herold, Jaspan 1986) where it helps in hepatic glucose production and output (Liljenquist *et al.* 1977). The secretion of glucagon from α -cells is regulated by the secretion from β -cells (Franklin *et al.* 2005, Weir *et al.* 1976) as well as by the sympathetic and parasympathetic nervous system as described above.

Figure 1.4



Tissue dependent post-translational processing of the proglucagon gene. The peptide precursor of the proglucagon protein, preproglucagon, undergoes cleavage resulting in the removal of the signal peptide present at the N-terminus. The remaining proglucagon gene undergoes post-translational modifications which are dependent on the tissue that the proglucagon protein are expressed in. In the α -cells of pancreatic islets, the proglucagon protein is processed by PC2 resulting in the production of glucagon. In the intestine, PC1/3 processes the proglucagon protein post-translationally resulting in the production of GLP-1 and GLP-2.

1.4.3: Somatostatin

Somatostatin is a hormone which inhibits insulin, growth hormone and glucagon secretion (Koerker *et al.* 1974, Brazeau *et al.* 1974, Gerich, Lovinger & Grodsky 1975). The somatostatin protein was initially thought to consist of 14 amino acids (Brazeau *et al.* 1973) however identification of the somatostatin coding gene (Shen, Rutter 1984, Shen, Pictet & Rutter 1982) lead to the discovery of several forms of somatostatin (Schally *et al.* 1976) with the larger 28 amino acid isoform being up to 5 times more potent at inhibiting insulin secretion than the 14 amino acid long peptide (Hadjidakis *et al.* 1986). When Asn⁵ is deleted the inhibitory effect on glucagon secretion is lost while D-Cys¹⁴-somatostatin was unable to inhibit insulin release (Luft, Efendić & Hökfelt 1978). The presence of somatostatin secreting δ -cells was first confirmed in humans in 1975 (Dubois *et al.* 1975). Acute

administration of somatostatin results in hypoglycaemia while long term administration leads to hyperglycaemia (Lins, Efendic 1976). Five different somatostatin receptors have been described (Rossowski, Coy 1994) with glucagon inhibition primarily being mediated through the SSTR2 receptor while SST5R is the main regulator of insulin inhibition (Strowski *et al.* 2000). Like both α and β -cells of the pancreatic islets, secretion of somatostatin from δ -cells is regulated by Ca^{2+} signalling and K_{ATP} channel depolarisation and thus blood glucose concentrations (Berts *et al.* 1996). As described above, pancreatic islets are closely related to the vascular system with β -cells being the first point of contact with the capillaries followed by α -cells. Further research into this architecture has shown that following on from the α -cells, the vascular system is directed towards the δ -cells thus suggesting that δ -cells have a paracrine effect on the secretion from β and α -cells (Stagner, Samols & Bonner-Weir 1988, Stagner, Samols 1992). This also allows the β -cells to signal to δ -cells, inhibiting somatostatin secretion through Urocortin3 (van der Meulen *et al.* 2015).

1.4.4: Pancreatic polypeptide (PP)

Pancreatic polypeptide (PP) was first described in 1975 as a 36 amino acid peptide with a molecular weight of 4240Da (Kimmel, Hayden & Pollock 1975). The gene sequence for the precursor protein from which PP is cleaved consists of a 95 amino acid peptide with PP being situated in the middle of this sequence (Leiter, Keutmann & Goodman 1984). The gene encoding PP is found on chromosome 17q21.1 and is 10kb apart from the peptide YY gene which is another closely related peptide (Hort *et al.* 1995). It appears that peptide YY arose from a duplication event of NPY while a second duplication of peptide YY resulted in PP. This is confirmed as peptide YY and PP have 70 and 55% sequence homology with NPY in humans (Hort *et al.* 1995). PP is mainly found in the pancreas and levels remain high for a longer duration than insulin postprandially (Adrian *et al.* 1976) and is secreted in response to ingestion of nutrients (Adrian *et al.* 1977). Obesity leads to lower PP levels (Glaser *et al.* 1988) and injection of PP in mice lead to reduced food intake (Asakawa *et al.* 1999).

1.4.5: Amylin

Amylin is another hormone which is secreted from the β -cells of the pancreas (Schmitz, Brock & Rungby 2004). It consists of a 67 amino acid propeptide which is co-localised with insulin in the secretory granules of β -cells and insulin production may regulate the production of amylin (Higham *et al.* 2000). Like insulin, amylin can reduce glucagon secretion while also reducing gastric emptying thus improving appetite control (Schmitz, Brock & Rungby 2004). The amylin peptide

itself is 37 amino acid long and is found to be aggregated in the pancreas of Type 2 Diabetes sufferers (Cooper *et al.* 1987).

1.5: G-protein coupled receptors (GPCRs)

GPCRs are the largest family of membrane bound proteins and are characterised by their 7 domains which span the membrane with the N-terminus being located extracellularly while the C-terminus extends into the cytoplasm (Horn *et al.* 2002, Isberg *et al.* 2015). There are roughly 800 GPCRs in the human genome (Isberg *et al.* 2015). The role of GPCRs is to allow the extracellular environment of the cell affect the processes and downstream signalling events intracellularly and the importance of this was recognised when Lefkowitz and Kobilka were awarded the Nobel Prize in Chemistry in 2012 for their discovery (Bockaert 2012). Intracellular signalling is carried out by an intact G-protein which is attached to inactive receptors on the cytosolic side of the cell membrane. The intact G-protein consists of 3 different subunits (α , β and γ) which are further split into different classes. The best characterised subunit is the α -subunit which has 4 different classes identified (G_{as} , G_{ai} , G_{aq} , and $G_{a12/13}$). The less well defined β -subunit has 5 classes while the γ -subunit has 12 different classes. The purpose of various different subunits is that there are a number of combinations of subunits and this can then be used to define the function of receptor activation (Layden, Durai & Lowe Jr. 2010).

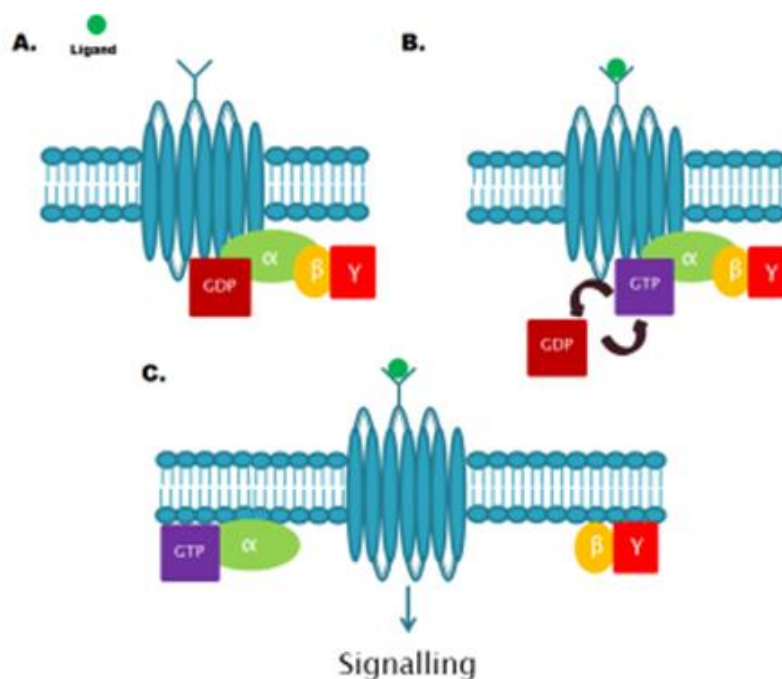
1.5.1: GPCR signalling cascades

The intact G-protein on the intracellular side of a GPCR is described as a heterotrimeric G-protein due to the composition of subunits from which it is made (Tuteja 2009). Upon ligand binding, the heterotrimeric G-protein undergoes a conformational change where the α -subunit dissociates from the β and γ -subunits leading to intracellular signalling (Layden, Durai & Lowe Jr. 2010). Dissociation of these subunits occurs when the C-terminal tail of the α -subunit catalyses the replacement of GDP, which is attached to the α -subunit in the resting heterotrimeric state, for GTP (Moran, Flatt & McKillop 2016). After this dissociation, both the α and $\beta\gamma$ -subunits have an effect on ion channels, phospholipases and adenylyl cyclases resulting in a biological effect due to the activation or inhibition of effectors by secondary messengers such as intracellular Ca^{2+} , cAMP, DAG and phosphoinositides (Lattin *et al.* 2007). G_{aq} and G_{ai} subunit signalling is controlled by regulators of G-protein signalling with GTPase activity (Tilley 2011). G_{as} signalling results in increased cAMP and protein kinase A (PKA) as a result (Tilley 2011, Moran, Flatt & McKillop 2016). Contrary to this, G_{ai} inhibits the production of cAMP and as a result there are increased in protein kinase C (PKC) (Moran, Flatt & McKillop 2016). $G_{a12/13}$ signalling has not been as

extensively studied however activation of this subunit leads to guanine nucleotide exchange factors (GEFs) known as RhoGEFs to be activated and ultimately the GTPase RhoA leading to a number of downstream signalling processes (Tilley 2011). The $G\beta\gamma$ -subunit is able to mediate a range of effectors such as PLC and adenylyl cyclase however these subunits need to be more extensively studied (Patel 2004).

1.5.2: GPCR mechanism of action

Figure 1.5.



Adapted from Layden, Durai & Lowe Jr. 2010.

A. In the receptors inactive form, a G-protein intact with all subunits present is attached to the receptor on the cytosolic side of the membrane. The G-protein is intact when the α -subunit is bound to the $\beta\gamma$ -subunits and GDP.

B. Upon receptor activation through ligand binding, the GDP bound to the G-protein is exchanged for GTP which forms a complex with the α -subunit.

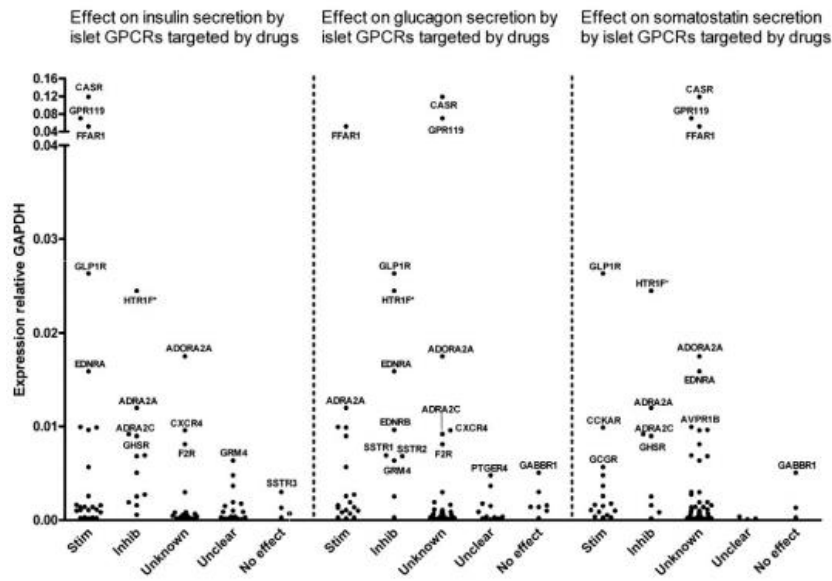
C. The α -subunit GTP complex dissociates from the $\beta\gamma$ -subunit. This triggers signalling processes inside the cell. The cycle is complete when the dissociated G-protein is swapped with an intact G-protein.

Upon extracellular ligand binding to the receptor, the intact intracellular G-protein attached on the intracellular side of the membrane undergoes a conformational change. GDP, which is attached to the $G\alpha$ -subunit, is replaced by GTP. This causes the $G\alpha$ -subunit to become dissociated from the $G\beta\gamma$ -subunit and leads to intracellular signalling. Due to the number of different combinations of subunits, there are a number of different signals which can be sent. The receptors themselves are split into 5 different families – rhodopsin, secretin, glutamate, adhesion and Frizzled/Taste2 (Layden, Durai & Lowe Jr. 2010). The largest GPCR family is the rhodopsin family with estimates that 80% of human GPCRs are classed as belonging to this group (Lagerstrom, Schioth 2008).

1.5.3: GPCRs as drug targets

There are a wide number of reviews discussing GPCRs as therapeutic drug targets with some estimates suggesting approximately 50% of all marketed drugs target a GPCR (Davies *et al.* 2008). In a 2006 analysis, over 20000 FDA approved drugs were grouped depending on the classification of their target (Overington, Al-Lazikani & Hopkins 2006). A total of 324 target families were identified with 26.8% of targets being classified as rhodopsin-like GPCRs. In a more recent study (Rask-Andersen, Almen & Schioth 2011) this was increased to approximately 36% of all FDA approved drugs (Figure 1.7). A particular area of interest for GPCRs is as therapeutic targets in Type 2 Diabetes. In the pancreas there are 293 GPCRs expressed in islets (Amisten *et al.* 2013). Many of these receptors are likely to be present in the gastrointestinal tract as large intestine, small intestine, pancreas and stomach were clustered together based on the expression of GPCRs (Regard, Sato & Coughlin 2008). The islet receptors have various roles in the pancreas however at there were 107 drugs found which increased insulin secretion while 184 drugs inhibited insulin secretion (Amisten *et al.* 2013). However the role of many of these GPCRs on pancreatic hormone secretion are still to be elucidated (Figure 1.6).

Figure 1.6



From (Amisten *et al.* 2013). A range of pancreatic GPCRs are targeted for the treatment of Type 2 diabetes. The graph above details the expression level of these receptors and their effects on insulin, glucagon and somatostatin secretion. The effects of many of the targeted GPCRs on hormonal secretion have not yet been elucidated.

Figure 1.7.



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From 193 proteins, 82 were GPCRs (42.5%). From the overall data set 36% of drugs targeted class A GPCRs. Over 21000 active agents were analysed with 1357 unique drugs being identified. (Rask-Andersen, Almen & Schioth 2011)

1.5.4: Monoatomic ion and macromolecule binding GPCRs

The monoatomic ions Ca^{2+} , H^+ , Mg^{2+} and Zn^{2+} are expressed ubiquitously throughout the body. Six different receptors which can be activated by these ions and macromolecules have been identified

as being expressed in human pancreatic islets (Amisten *et al.* 2013). Ca^{2+} and H^+ can activate the calcium-sensing receptor (CaSR) and the ovarian cancer gene receptor 1 (OGR1) respectively (Wei *et al.* 2015). Ca^{2+} has also been shown to activate the GABA_B receptor (Wise *et al.* 1999) and the mGluR receptors (Kubo, Miyashita & Murata 1998) however the activation of mGluR by Ca^{2+} receptors has been questioned (Nash *et al.* 2001). Mg^{2+} also appears to activate the CaSR and this receptor is expressed in a range of tissues throughout the body including the intestine, placenta, cartilage-forming cells and a range of cells in the bone (Brown, MacLeod 2001). Zn^{2+} has been shown to activate the zinc-sensing receptor ZnR which is also known as GPR39 (Cohen *et al.* 2012), toll-like receptor 4 (TLR4) (Liang, Dempski & Burdette 2016), the SLC30 and SLC39 families of zinc transporters (Jeong, Eide 2013) as well as the P2X4 and P2X2 receptors, the second of which is also activated by CuCl_2 (Xiong *et al.* 1999). H^+ has also been shown to activate GPR4, GPR68 and GPR132 in the pancreas (Amisten *et al.* 2013)

1.5.5: G-protein coupled receptor 39 (GPR39)

As stated above, GPR39 is also called ZnR and it is the zinc-sensing receptor that has been researched the most. Both GPR39 and Zn^{2+} have been shown to be present in the islets of the pancreas (Amisten *et al.* 2013) while other tissues that express GPR39 include the liver, kidney, intestines and white adipose tissue (Egerod *et al.* 2007b). Previous work looking at GPR39 has shown that activation of this receptor results in insulin secretion both in vitro and in vivo (Moran *et al.* 2016a). This confirmed earlier work which demonstrated the insulinotropic effect of GPR39 in both age-dependent and diet-induced insulin resistant mice by knocking out GPR39 (Tremblay *et al.* 2009, Holst *et al.* 2009b). A number of different $\text{G}\alpha$ subunits attach to GPR39 and as a result it affects a number of different signalling pathways as it has demonstrated an ability to increase intracellular calcium, cAMP (Moran *et al.* 2016a) as well as IP_3 turnover (Holst *et al.* 2007a). GPR39 was originally thought to be stimulated by obestatin (Zhang *et al.* 2005a), however further studies have concluded that this is not the case (Holst *et al.* 2007a). Two forms of the GPR39 receptor have been identified, the active GPR39-1a form and an inactive truncated form which is known as GPR39-1b and is a 5 transmembrane domain truncated form of the protein (Egerod *et al.* 2007b, Yasuda, Ishida 2014).

1.5.6: Cannabinoid receptors

The first cannabinoid receptor to be recognised was the CB_1 receptor (Matsuda *et al.* 1990). This was followed by the identification of the CB_2 receptor (Munro, Thomas & Abu-Shaar 1993). Both CB_1 and CB_2 are attached to a $\text{G}\alpha_i$ subunit on the cytosolic side of the membrane (Howlett 2002),

however CB₁ can also attach to Gα_s and Gα_q proteins (Howlett *et al.* 2002). CB₁ is highly expressed in the brain, with lower expression levels found throughout the body in tissues such as the liver, pancreas and muscle while CB₂ is expressed in neurons and immune cells (Mackie 2008). Other putative cannabinoid receptors have been suggested such as vanilloid type 1 receptor (TRPV1) and GPR55 (Aizpurua-Olaizola *et al.* 2017). CB₁ activation by rimonabant has positive effects on weight loss in obese people, however due to the detrimental side effects on people with mental disorders this drug was removed as a therapeutic aid (Moreira, Crippa 2009). CB₂ activation has been linked with reduced inflammation however it might also be a trigger for increased tissue damage (Pacher, Mechoulam 2011).

1.5.7: G-protein coupled receptor 55 (GPR55)

GPR55 is a putative cannabinoid receptor which is expressed in a range of tissues such as pancreatic islets (Amisten *et al.* 2013), gastrointestinal tract, brain (Ryberg *et al.* 2007) and white adipose tissue (Beltowski 2012). Lysophosphatidylinositol (LPI) was the first compound to be identified as a GPR55 agonist (Oka *et al.* 2007), however this compound was later shown to stimulate ovarian cancer cell growth (Goldsmith *et al.* 2011). Whilst there is less known about GPR55, the insulinotropic effects of GPR55 have previously been demonstrated (Romero-Zerbo *et al.* 2011, McKillop *et al.* 2013, McKillop *et al.* 2016) and GPR55 has been shown to be activated by a range of synthetic and endogenous fatty acids (McKillop *et al.* 2013).

1.5.8: G-protein coupled receptor 119 (GPR119)

GPR119 is a class A rhodopsin-like GPCR (Fredriksson *et al.* 2003c). GPR119 is expressed in both the L-cells of the small intestine and pancreatic islets and it has been described as the most expressed cannabinoid receptor in the β-cells of the pancreas (Kebede *et al.* 2009, Amisten *et al.* 2013). Activation of GPR119 present in the β-cells of the pancreas has resulted in increased glucose-stimulated insulin release (Moran *et al.* 2014a) and it has also been shown to increase PYY and incretin hormone secretion from the intestine (Chu *et al.* 2008). The endogenous ligand OEA was the first GPR119 agonist to be identified (Overton *et al.* 2006) however this ligand has also been shown to activate GPR55 (McKillop *et al.* 2013). GPR119 activation increases cAMP and is bound to the Gα_s subunit (Chu *et al.* 2007b, McKillop *et al.* 2013). Insulin secretion mediated by GPR119 is dependent on glucose while GLP-1 and GIP release are not (Lan *et al.* 2012).

1.5.9: Free Fatty Acid Receptors (FFAR)

Of the 293 identified GPCRs in the human islet, 99 receptors were activated by 87 small organic molecules (Amisten *et al.* 2013). Small organic molecules were defined as lipids, free fatty acids, nucleotides, steroids, eicosanoids and amino acids. The number of activating molecules is less than the number of receptors activated as 35 small organic molecules were able to activate 2 or more receptors. The highest expressed receptor which is activated by fatty acids is GPR40. GPR40 expression levels were over 100 times higher than those seen for GPR41 and GPR43. These receptors are activated by various fatty acids but are dependent on the triglyceride chain length with short chain fatty acids having six or less carbons, medium chain fatty acids having 6 to 12 carbons and long chain fatty acids being longer than medium chain fatty acids (Miyamoto *et al.* 2016). Fatty acid receptors are essential as some of the essential fatty acids which cannot be synthesised in the human body signal through these receptors.

1.5.10: G-protein coupled receptor 40 (GPR40)

As stated above, GPR40 is the most abundant free fatty acid receptor in pancreatic islets and it is activated by both medium and long chain fatty acids (Briscoe *et al.* 2003). The sequence of this receptor is conserved across various species (Itoh *et al.* 2003a) and it has been shown to signal through both the $G\alpha_q$ and $G\alpha_s$ pathways (Briscoe *et al.* 2003, Welters *et al.* 2006). Both DHA and EPA are the most potent agonists for GPR40 however these agonists can also activate GPR120 (Moran *et al.* 2014b, Miyamoto *et al.* 2016). GPR40 is expressed in the intestine tastes buds and central nervous system (Ma *et al.* 2007, Edfalk, Steneberg & Edlund 2008, Cartoni *et al.* 2010, Sykaras *et al.* 2012a). Activation of GPR40 increases both incretin and insulin secretion and is a potential therapeutic target in Type 2 Diabetes (Itoh *et al.* 2003a, Edfalk, Steneberg & Edlund 2008). In fact a selective GPR40 agonist made it to stage 3 clinical trials for the treatment of Type 2 Diabetes however this was stopped at this stage due to concerns over liver toxicity (Kaku *et al.* 2015).

1.5.11: G-protein coupled receptor 41 (GPR41)

GPR41 is activated by short chain fatty acids such as formate, propionate, butyrate, and pentanoate with pentanoate being the most potent out of these agonists (Brown *et al.* 2003). It has been shown to increase IP_3 and intracellular calcium and is attached to a $G\alpha_i$ subunit (Le Poul *et al.* 2003). Activation of GPR41 has been shown to increase leptin levels and knockout of this receptor has been linked to a variety of inflammatory disorders such as obesity and asthma (Ang, Ding 2016). The ligands for GPR41 are produced endogenously in the intestine through the fermentation of fibre

which has been ingested (Tazoe *et al.* 2008). GPR41 is expressed in adipose tissue, pancreas, spleen, lymph nodes, bone marrow, and peripheral blood mononuclear cells (Ang, Ding 2016). Due to the different tissue GPR41 is expressed in, it can be hard to determine the function of this receptor. However knockout of GPR41 leads to hypoglycaemia and increased insulin secretion (Veprik *et al.* 2016, Tang *et al.* 2015). Interestingly GPR41 agonists have been shown to increase the secretion of the incretin hormone GLP-1 which is in contrast to knockout models looking at GPR41 effects on glucose homeostasis (Tolhurst *et al.* 2011b).

1.5.12: G-protein coupled receptor 43 (GPR43)

Like GPR41, GPR43 is activated by short chain fatty acid agonists (Brown *et al.* 2003, Le Poul *et al.* 2003). It is found in the intestine and activation of this receptor has been shown to increase PYY secretion from the L cells of the small intestine (Karaki *et al.* 2006). GPR43 can bind to both $G\alpha_i$ and $G\alpha_q$ subunits and as a result there is some confusion over the role that this receptor plays in various biological processes. For instance, one study found that GPR43 activation can both enhance and inhibit glucose stimulated insulin secretion (Priyadarshini *et al.* 2015). Although the sequence of GPR43 is highly conserved between the human and mouse genomes (Nilsson *et al.* 2003), different GPR43 agonists have shown to have differing responses in each species (Priyadarshini *et al.* 2015). Therefore there may be limitations in using murine models when assessing the effectiveness of GPR43 as a therapy for Type 2 Diabetes.

1.5.13: G-protein coupled receptor 84 (GPR84)

GPR84 is another receptor which is activated by fatty acid ligands. In particular, GPR84 is activated by medium chain fatty acids with a triglyceride chain length of 9-14 carbons (Wang *et al.* 2006). GPR84 was first described in 2001 and the gene which codes for this receptor is located on chromosome 12q13.13 in humans (Wittenberger, Schaller & Hellebrand 2001). The GPR84 protein is 396 amino acids long and was also known as EX33 which was identified as being expressed in bone marrow, lung and peripheral blood leukocytes (Yousefi *et al.* 2001). The first study to identify GPR84 agonists identified decanoic acid (10 carbon chain), undecanoic acid (11 carbon chain) and lauric acid (12 carbon chain) as potent stimuli of GPR84 (Wang *et al.* 2006) however new agonists are still being discovered (Zhang *et al.* 2016)

1.5.14: G-protein coupled receptor 120 (GPR120)

GPR120 is a class A rhodopsin-like GPCR (Fredriksson *et al.* 2003c) that is also classified as a free fatty acid receptor (Amisten *et al.* 2013). Like GPR43, GPR120 is activated by both medium and long chain fatty acids despite having only 10% sequence homology (Miyamoto *et al.* 2016). The receptor appears to signal through the $G\alpha_q$ pathway, however further research is required to elucidate which other pathways GPR120 activates (Miyamoto *et al.* 2016). Activation of GPR120 by fatty acid agonists increases glucose stimulated insulin secretion from the pancreas (Moran *et al.* 2014b) and GLP-1 and CCK secretion from the intestine (Hirasawa *et al.* 2005a, Sidhu *et al.* 2000). This suggests that GPR120 plays an important role in glucose homeostasis and is an attractive therapeutic target for the treatment of Type 2 Diabetes. This is confirmed by the expression of GPR120 in a range of tissues which are affected in metabolic disorders such as the intestine, pancreas, adipocytes and immune cells (Miyamoto *et al.* 2016).

1.5.15: GPCRs activated by peptides

Of the 293 receptors present in the islet, 110 were identified as being activated by a peptide with 178 peptides being agonists for islet GPCRs and 80 peptides activating 2 or more receptors (Amisten *et al.* 2013). Advances in crystallisation techniques means that this under targeted area of GPCR may become thoroughly researched using structural based screens for ligands (Ranganathan *et al.* 2017).

1.5.16: GLP-1 receptor (GLP1R)

In humans the GLP1R is a 463 amino acid receptor which activates multiple signalling pathways (Dillon *et al.* 1993). It is expressed in both the pancreas and the brain (Dillon *et al.* 1993, Cork *et al.* 2015). GLP1R activation increases both insulin and somatostatin release from the pancreas (Heller, Aponte 1995) while reducing glucagon secretion (Hare *et al.* 2009). The GLP1R agonist exenatide has been used as a therapy in Type 2 Diabetes since 2005 and has been shown to have positive effects on glucose homeostasis and weight loss (Kendall *et al.* 2005, Buse *et al.* 2010). Another GLP1R agonist, Liraglutide, is also clinically available and has the same positive effects as exenatide (Shyangdan *et al.* 2011). GLP-1 mimetics are desirable GLP1R agonists as they have a longer half-life than GLP-1 (Agerso *et al.* 2002). Activation of this receptor may lead to acute pancreatitis (Anderson, Trujillo 2010) or the formation against the drug itself, which is more likely with slower release exenatide (Russell-Jones 2010).

1.5.17: GIP receptor (GIPR)

The gene that codes for the GIPR is found on chromosome 19q13.2-q13.3 (Stoffel *et al.* 1995) and is expressed in the gut, adipose tissue, heart, pituitary gland and brain (Usdin *et al.* 1993). The GIPR is a 455 amino acid long protein with 44% sequence homology with the glucagon receptor (Brubaker, Drucker 2002). GIPR expression has been shown to be reduced in the islets of diabetic rats (Lynn *et al.* 2001). GIPR activation leads to increases in both cAMP and insulin secretion in pancreatic β -cells (Szecowka *et al.* 1982) while in normal glucose concentrations GIPR activation results in increased glucagon secretion (Meier *et al.* 2003).

1.5.18: Glucagon receptor (GCGR)

The human GCGR gene can be found on chromosome 17q25 and with a coding region of over 5.5kb pairs and encodes a 477 amino acid long protein (Lok *et al.* 1994, Menzel *et al.* 1994). GCGR is a class B GPCR which is expressed in various tissues within the body including the pancreas, liver, brain, heart and kidney (Brubaker, Drucker 2002, Siu *et al.* 2013). A mutation in the GCGR gene has been implicated in Type 2 Diabetes in people of European descent, however this mutation was not found in diabetic individuals from Japan (Hager *et al.* 1995, Fujisawa *et al.* 1995). An extracellular missense mutation in GCGR resulted in reduced glucagon affinity for GCGR, hyperglucagonemia and α -cell hyperplasia (Zhou *et al.* 2009). When the body is in a fasting state, glucagon binds to the GCGR and this leads to hepatic glucose release (Siu *et al.* 2013). The GCGR is bound to both the $G\alpha_q$ and $G\alpha_i$ subunits through which it increases intracellular calcium through the PLC pathway (Xu, Xie 2009). Interestingly, despite the cAMP pathway inhibition shown by the $G\alpha_i$ subunit, the GCGR has also been shown to increase cAMP through $G\alpha_s$ binding (Iyengar *et al.* 1988).

1.5.19: G-protein coupled receptor 75 (GPR75)

The first recognised GPR75 agonist was the proinflammatory chemokine CCL5/RANTES (Ignatov *et al.* 2006). Recently a second agonist has been identified, which is known as 20-HETE (Garcia *et al.* 2017). It is suspected that GPR75 is attached to a $G\alpha_q$ subunit intracellularly as GPR75 activation leads to increased IP3 and calcium levels intracellularly (Ignatov *et al.* 2006, Garcia *et al.* 2017). GPR75 is widely expressed throughout the body with high levels of expression seen in the brain, endocrine glands and reproductive organs (Garcia *et al.* 2017). GPR75 has also been shown to be present in the islets of the pancreas and its activation by CCL5 has increased insulin secretion and glucose tolerance (Liu *et al.* 2013).

1.5.20: Neuropeptide receptors

The human islet has various neuropeptide sensing receptors present which includes Y1, Y4, Y5 and GPR83 which are activated by neuropeptide Y (NPY), pancreatic polypeptide (PP) and PYY (Amisten *et al.* 2013). These receptors are expressed throughout the body including the brain and spinal cord (Brothers, Wahlestedt 2010, Muller *et al.* 2013). NPY has been shown to inhibit insulin secretion while there is some confusion over its role on glucagon secretion as it increases secretion in healthy rats but suppresses it in diabetic rats (Ponery, Adeghate 2000, Schwetz, Ustione & Piston 2013). PYY binds to all of the above receptors except Y5 but also binds to the Y2 receptor (Sah *et al.* 2007). PP binds to the Y1, Y2, Y4 and Y5 receptors and has been shown to inhibit insulin secretion in rats (Amisten *et al.* 2013, Murphy *et al.* 1981).

1.6: Aims and objectives

The effects of GPCR agonists such as GLP-1 on the pancreas have been extensively studied. It is thought that lipid, protein and carbohydrate sensing GPCRs are present in the small intestine in order to signal the presence of nutrients. The localisation and function of GPCRs in the small intestine is not as well established as those present in the pancreas. Fatty acid sensing GPCRs such as GPR40, GPR41 and GPR43 have been of particular interest in this research area. There are currently limited studies which have assessed the ability of both endogenous and synthetic agonists of other GPCRs to control glucose homeostasis through the regulation of gastrointestinal hormone secretion. There are also limited studies investigating the effects of GPCR agonists as a combination therapy with DPP-IV inhibitors.

The main aims of the research project are as follows:

- Evaluate the ability of agonists of a range of GPCRs (GPR39, GPR55, GPR75, GPR119 and GPR120) to increase GLP-1, GIP and insulin secretion from intestinal (GLUTag and pGIPneo STC-1) and pancreatic (BRIN-BD11) cell lines as well as lean and diabetic mouse models
- Determine the expression and localisation of GPCRs and gastrointestinal hormones (GLP-1, GIP and PYY) in a range of intestinal cell lines as well as lean and diabetic mouse models using qPCR and immunohistochemistry
- Examine the acute effects of GPCR agonist and DPP-IV inhibitor combination therapy on glucose homeostasis in lean mouse models
- Investigate the long term effects of GPR55 agonist Abn-CBD and GPR119 agonist AS1269574 monotherapy and combination therapy with Sitagliptin in a diabetic animal model

1.6.1: Hypothesis

The hypothesis of this thesis is that the GPCRs investigated are present in the enteroendocrine cells of the intestine and can be targeted to increase incretin hormone and insulin secretion and improve glucose homeostasis *in vitro* and *in vivo* for the treatment of Type 2 Diabetes.

Chapter 2

Materials and Methods

2.1: Tissue culture

2.1.1: Materials

RPMI 1640 medium, 1x Dulbecco's modified Eagle's medium (DMEM), 4.5x DMEM and foetal bovine serum (FBS), penicillin/streptomycin (100U/ml; 0.1mg/ml), 10x trypsin/EDTA and Hanks Buffered Saline Solution (HBSS) were all purchased from Gibco Life Technologies (Paisley, Strathclyde, UK). HEPES, sodium chloride (NaCl), potassium chloride (KCl), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), sodium hydroxide (NaOH), dimethylsulphoxide (DMSO), geneticin (G418), bovine serum albumin (BSA) and hydrogen peroxide (H_2O_2) were sourced from Sigma-Aldrich (Poole, Dorset, UK). Sterile, flat bottom, 96-well tissue culture plates were obtained from Nunclon (Roskilde, Denmark).

2.1.2: Culture of clonal BRIN-BD11 cells

The effects of GPCR agonists on insulin secretion and cell viability were investigated using the insulin secreting BRIN-BD11 cell line. In order to establish the insulin secreting pancreatic BRIN-BD11 cell line, RINm5F cells were fused with New England Deaconess Hospital (NEDH) rat pancreatic beta cells via electroporation (McClenaghan *et al.* 1996). Cells (passages 15-45) were maintained in RPMI-1640 media with 1% antibiotics (v/v), 10% FBS (v/v) and a glucose concentration of 11.1mM. Cells were grown in 20ml of media in 75cm³ vented sterile tissue culture flasks at 37°C in 5% CO₂ and 95% air inside a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK). Cell stocks, containing 1 million BRIN-BD11 cells, were cryopreserved in 1ml of freezing medium containing 80% FBS (v/v), 10% RPMI-1640 (v/v), 10% DMSO (v/v) and stored in 1.5ml cryovials (Sterilin, Houslow, UK). Cryovials containing stocks were stored at -20°C for 4 hr before transfer to -80°C until long-term storage in liquid nitrogen.

Confluent cells (75-90%) were harvested by washing in 10ml of HBSS and incubated with pre-warmed 0.025% (w/v) trypsin/EDTA at 37°C for 5 min. Confirmation of detachment was performed by visual inspection using a phase contrast microscope (Zeiss, Germany). Cells were resuspended in 5ml of RPMI-1640 media, transferred to a 20ml sterilin tube (Sterilin, Houslow, UK) and centrifuged at 900rpm for 5 mins using a bench centrifuge (MSE Mistral 200, RHYS International, Manchester, UK). Supernatant was decanted and the cell pellet resuspended in a known volume of media. An aliquot of cell suspension (100µl) was stained by adding to trypan blue (100µl, 1:2 dilution), mixed thoroughly and added to a Neubauer haemocytometer (Scientific Supplies Co, Middlesex, UK) for counting.

2.1.3: Culture of GLUTag cells

GLUTag cells (a gift courtesy of Professor D. J. Drucker, University of Toronto, Canada) were developed from nude transgenic mice producing a glucagon expressing neuroendocrine carcinoma due to the presence of a glucagon gene simian virus-40 large T-antigen (GLUTag) fusion gene as described previously (Drucker *et al.* 1992). This cell line was utilised to test the effect of GPCR activation on GLP-1 secretion and cell viability *in vitro*. Cells were cultured in DMEM containing 5.5mM glucose, 2mM L-glutamine, 10% (v/v) FBS and antibiotics (penicillin (100U/ml), streptomycin (0.1mg/l)). Cell stocks, containing 1 million cells, were cryopreserved in 1ml of freezing medium containing 80% FBS (v/v), 10% DMEM (v/v), 10% DMSO (v/v) and stored in 1.5ml cryovials (Sterilin, Houslow, UK). Cryovials containing stocks were stored at -20°C for 4 hr before transfer to -80°C until long-term storage in liquid nitrogen.

Confluent cells (70-90%) were harvested by washing in 10ml of HBSS and incubated with pre-warmed 0.025% (w/v) trypsin/EDTA at 37°C for 5 min. Confirmation of detachment was performed by visual inspection using a phase contrast microscope (Zeiss, Germany). Cells were resuspended in 5ml of DMEM medium, transferred to a 20ml sterilin tube (Sterilin, Houslow, UK) and mixed up and down vigorously by pipetting. For counting, cells were pelleted by centrifugation at 900rpm for 5 mins using a bench centrifuge (MSE Mistral 200, RHYS International, Manchester, UK). Supernatant was decanted and the cell pellet resuspended in a known volume of media. An aliquot of cell suspension (100µl) was stained by adding to trypan blue (100µl, 1:2 dilution), mixed thoroughly and added to a Neubauer haemocytometer (Scientific Supplies Co, Middlesex, UK).

2.1.4: Culture of pGIPneo STC-1 cells

pGIPneo STC-1 cells were a kind gift from Dr. Burton Wice (Washington University in St. Louis, USA) with permission from Professor Douglas Hanahan (École Polytechnique Fédérale de Lausanne, CH). These cells were developed via the transfection of native STC-1 cells with a plasmid (pGIPneo) which encoded for neomycin phosphotransferase and selected cells containing the GIP promoter. Due to the increased number of cells containing the GIP promoter, pGIPneo STC-1 cells were used to examine the *in vitro* effects of GPCR activation on GIP secretion and cell viability. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25mM glucose, 2mM L-glutamine, 10% (v/v) FBS and antibiotics (penicillin (100U/ml), streptomycin (0.1mg/l) and geneticin (400 µg/mL)). Cell stocks, containing 1 million cells, were cryopreserved in 1ml of freezing medium containing 80% FBS (v/v), 10% DMEM (v/v), 10% DMSO (v/v) and stored in 1.5ml cryovials (Sterilin, Houslow, UK). Cryovials containing stocks were stored at -20°C for 4 hr before transfer to -80°C until long-term storage in liquid nitrogen. Confluent cells (70-90%) were harvested and cells were counted as described in section 2.1.4.

2.1.5: Culture of STC-1 cells

STC-1 cells were a kind gift from Dr. Burton Wice (Washington University in St. Louis, USA) with permission from Professor Douglas Hanahan (École Polytechnique Fédérale de Lausanne, CH). STC-1 cells were developed from a RIP1Tag2/RIP2PyST1 mouse which developed intestinal neuroendocrine tumours (Grant *et al.* 1991). STC-1 cells were used to investigate the localisation of GPCRs in PYY immunoreactive cells. STC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25mM glucose, 2mM L-glutamine, 17.5% (v/v) FBS and antibiotics (penicillin (100U/ml) and streptomycin (0.1mg/l)). Cell stocks, containing 1 million cells, were cryopreserved in 1ml of freezing medium containing 80% FBS (v/v), 10% DMEM (v/v), 10% DMSO (v/v) and stored in 1.5ml cryovials (Sterilin, Houslow, UK). Cryovials containing stocks were stored at -20°C for 4 hr before transfer to -80°C until long-term storage in liquid nitrogen. Confluent cells (70-90%) were harvested and cells were counted as described in section 2.1.4.

2.1.6: Acute insulin secretory tests in BRIN-BD11 cells

Confluent BRIN-BD11 (70-90%) cells were cultured and harvested as described in section 2.1.2. Cells were subsequently seeded into 24 well plates (Nunc, Roskilde, DK) with each well containing 150,000 cells and 1ml RPMI-1640 media. Upon seeding cells were incubated at 37°C in 5% CO₂ and 95% air overnight to allow for the development of monolayers. Before test solutions were added, media was decanted and cells were initially incubated with 1ml of 1.1mM glucose in Krebs Ringer Bicarbonate Buffer (KRBB, 4.7mM KCl, 115mM NaCl, 1.28mM CaCl₂·2H₂O, 25mM NaHCO₃, 0.1% BSA (w/v), 1.2mM KH₂PO₄, 1.2mM MgSO₄·7H₂O, 20mM HEPES (pH 7.4)) for 40 mins at 37°C in 5% CO₂ and 95% air. Following this incubation, 1.1mM glucose was decanted and cells were incubated with varying concentrations (10⁻¹² to 10⁻⁴M) of GPCR agonists in both 5.6mM and 16.7mM glucose in KRBB for 20 mins at 37°C in 5% CO₂ and 95% air. Supernatant (950µl) of test solutions were collected subsequent to this incubation and stored at -20°C until insulin measurement by radioimmunoassay or LDH assay.

2.1.7: Acute incretin secretory tests in GLUTag and pGIPneo STC-1 cells

Confluent cells (70-90%) were cultured and harvested as described in sections 2.1.3 and 2.1.4. Cells were subsequently seeded into 24 well plates with each well containing 150,000 cells and 1ml of culture media. Cells were mixed vigorously up and down via pipetting before seeding to allow for the development of monolayers and incubated at 37°C in 5% CO₂ and 95% air for 48 hrs. Before test solutions were added, media was decanted and cells were initially incubated with 1ml of 1.1mM glucose in KRBB for 1 hr at 37°C in 5% CO₂ and 95% air. Following this incubation, 1.1mM

glucose was decanted and cells were incubated with varying concentrations (10^{-12} to 10^{-4} M) of GPCR agonists in 2mM glucose in KRBB for 2 hrs at 37°C in 5% CO₂ and 95% air. Supernatant (950µl) of test solutions were collected subsequent to this incubation and stored at -20°C until hormone measurement by ELISA.

2.1.8: Alamar Blue Assay

BRIN-BD11 cells were harvested as described above (section 2.1.2) and seeded into 96-well plates (Nunc, Roskilde, DK) at a density of 40000 cells per well. Cells were incubated overnight at 37°C in 5% CO₂ and 95% air. After overnight incubation, media was decanted and test solutions consisting of varying concentrations (10^{-12} to 10^{-4} M) of GPCR agonists were added in both 5.6mM and 16.7mM glucose in KRBB and incubated for 20 mins at 37°C in 5% CO₂ and 95% air. Test solutions were subsequently decanted and 10µl of FluoroFire-Blue ProViaTox (Molecutools, Dublin, IRL) was added and cells were incubated at 37°C, 5% CO₂ and 95% air for 5 hrs. Fluorescence excitation and emission were measured using the FlexStation 3 (Molecular Devices, CA, USA) at 560nm and 590nm respectively.

2.1.9: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

BRIN-BD11, GLUTag and pGIPneo STC-1 cells were harvested as described above (sections 2.1.2, 2.1.3 and 2.1.4) and seeded into 96-well plates (Nunc, Roskilde, DK) at a density of 40000 cells per well. BRIN-BD11 cells were incubated overnight at 37°C in 5% CO₂ and 95% air. After overnight incubation, media was decanted and test solutions consisting of varying concentrations (10^{-12} to 10^{-4} M) of GPCR agonists were added in both 5.6mM and 16.7mM glucose in KRBB and incubated for 20 mins at 37°C in 5% CO₂ and 95% air. GLUTag and pGIPneo STC-1 cells were incubated for 48 hrs at 37°C in 5% CO₂ and 95% air. Subsequent to this incubation, media was decanted and test solutions consisting of varying concentrations (10^{-12} to 10^{-4} M) of GPCR agonists were added in 2mM glucose for 2 hrs at 37°C in 5% CO₂ and 95% air. Following incubation, test solutions were decanted and 0.5mg/ml MTT in culture media was added for 2 hrs and cells were incubated at 37°C in 5% CO₂ and 95% air. MTT was decanted and DMSO and cells were incubated for 5 mins on a horizontal orbital microplate shaker (Titramax 1000, Heidolph Instruments, Schwabach, DE). Fluorescence excitation and emission were measured using the FlexStation 3 (Molecular Devices, CA, USA) at 570nm and 630nm respectively.

2.2: Insulin Radioimmunoassay (RIA)

2.2.1: Materials

Thimerosal, iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril), BSA, activated charcoal, sequencing grade TFA, dextran T-70 and bovine insulin were purchased from Sigma-Aldrich (Poole, Dorset, UK). Dichloromethane (CH₂Cl₂) and HPLC grade acetonitrile were sourced from Rathburn (Walkersburn, UK). Radiolabelled sodium iodide (Na¹²⁵I) was supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK). Rat insulin standards were purchased from Novo Industria (Copenhagen, Denmark). All other chemicals used were obtained from BHD chemicals (Poole, Dorset, UK).

2.2.2: Preparation of iodinated bovine insulin

In order to coat microcentrifuge tubes (Sarstedt, DE) with a layer of iodogen, dichloromethane (100 μ g/ml) was used to dissolve iodogen. Dissolved iodogen was then added to tubes in 100 μ l aliquots before evaporation using a gentle stream of helium to ensure the iodogen layer was uniform. Coated iodogen reaction tubes were then used and iodination was carried out in a designated controlled area with both lead and Perspex shielding. A 20 μ l solution of bovine insulin (125 μ g/ml in 500mM sodium phosphate buffer, pH 7.4) and 5 μ l of Na¹²⁵I (100mCi/ml) were added to the coated iodogen tubes and tubes were agitated 3-4 times per min while being cooled on ice between agitations. This continued for 15 mins. In order to stop the reaction, the mixture was transferred from the reaction tube into a fresh microcentrifuge tube, with the reaction tube being washed with 500 μ l of 50mM sodium phosphate buffer. This buffer was also transferred to the new microcentrifuge tube. The iodination mixture was kept cool on ice until reverse-phase high performance liquid chromatography (RP-HPLC) separation of bound and unbound fractions.

A Spectra Series P200 TSP HPLC gradient pump system (CA, USA) with a Vydac C-8 analytical column (4.6 x 250mm, Phenomenex, Macclesfield, UK) was used to separate fractions using RP-HPLC. Column equilibrium was achieved using 0.12% (v/v) TFA/H₂O and eluted with 0.1% (v/v) TFA in 70% acetonitrile/H₂O from 0-40% over 10 mins, from 40-80% over 40 mins and 80-100% over 10 mins. Fractions were collected using a LKB2112 Redirac fraction collector (Bromma, S) in 72 x 12mm polypropylene tubes (Sarstedt, DE). From each 1ml fraction, 5 μ l aliquots were transferred to plastic LP3 tubes (Sarstedt, DE) and counts per min (CPM) measured using a gamma counter (1470 Wizard multigamma counter, PerkinElmer, USA). Fractions with the highest CPM underwent an antibody binding test. Fractions with similar binding were subsequently pooled and double diluted using 40mM sodium phosphate buffer (pH 7.4) containing 1% BSA (w/v), 1.2mg/ml

thiomersal and stored at 4°C. A typical RP-HPLC elution profile of the iodination reaction with CPM/μl versus time (min) is displayed in Fig. 2.1.

2.2.3: Determination of insulin by RIA

The determination of insulin content of both *in vitro* and *in vivo* samples was achieved using a modified dextran-coated charcoal RIA as previously described (Flatt, Bailey 1981). Stock assay buffer was prepared using 40mM disodium hydrogen orthophosphate with 0.3% NaCl (w/v) and 0.02% thiomersol (w/v). This buffer was titrated with 40mM sodium dihydrogen orthophosphate until pH 7.4 was observed. Working assay buffer consisted of stock assay buffer supplemented with 0.5% BSA (w/v) and was used to dilute guinea-pig anti-porcine insulin antibody (1:35000 to 1:55000 depending on the binding test), rat insulin standards and bovine insulin labelled with I¹²⁵. A standard curve (0.039-20ng/ml) for rat insulin standards was prepared by serial dilution and each standard was assayed in triplicate. Diluted antibody (100μl) was added to each standard or unknown sample. Total and non-specific binding tubes received no antibody. Samples were incubated for 24 hrs before bovine insulin labelled with I¹²⁵, which was diluted in working assay buffer to achieve 10,000 CPM/100μl, was added to all tubes including total and non-specific binding. Samples were incubated for a further 24 hrs at 4°C. Working assay buffer was used to dilute dextran T-70 coated charcoal 1:5 and 1ml of this was added to all tubes except the total tubes for 20 mins at 4°C. Following this 20 min incubation, tubes were centrifuged for 20 mins at 2,500rpm and 4°C using a Model J-6B Centrifuge (Beckman Instruments, UK). Following centrifugation, the supernatant was decanted with unbound I¹²⁵ labelled insulin fraction remaining as a black pellet. The CPM of this pellet was measured using the gamma counter. There was an inverse correlation between counts bound to the antibody (total CPM minus CPM bound to charcoal) to the concentration of insulin in standards or unknown samples. The rat insulin standard curve was constructed with spline curve fitting algorithm with unknown samples being interpolated from the curve. The rat insulin standard stock of 1 ng/ml insulin is equal to 173 pM and conversion from ng/ml to pM was performed by multiplication by a factor of 173. A typical rat insulin standard curve is shown with CPM versus log insulin concentrations in Fig. 2.2.

2.3: Enzyme-linked immunosorbent assay (ELISA)

2.3.1: Materials

Multi species GLP-1 total ELISA and rat/mouse GIP total ELISA kits were purchased from Merck Millipore (Watford, UK).

2.3.2: Measurement of GLP-1

Multi species GLP-1 total ELISA kits were used to quantify the GLP-1 content of both plasma and cell culture supernatant samples. This is a sandwich based ELISA which measures both the 7-36 and 9-36 forms of GLP-1. For cell supernatant samples, 50µl of supernatant collected in section 2.1 was used. For plasma samples, 20µl of plasma was diluted in 30µl of assay buffer. Each kit contained one 96 well plate.

Components of the ELISA were pre-warmed to room temperature before commencing the assay as per manufacturer's protocol. A 1000pM GLP-1 standard underwent a series of dilutions in order to obtain a standard curve ranging from 4.1 to 1000pM. Quality control 1 and quality control 2 were reconstituted in µQ H₂O with excess kept at -20°C. Blank, standard and quality control wells of the 96 well plate received 50µl of matrix solution. Blank and unknown sample wells received 50µl of assay buffer. Following plate and sample preparation, 50µl of standards, quality controls and unknown samples were added to the appropriate wells. Blank, standard and quality control wells were tested in duplicate while unknown samples were assayed in triplicate. The plate was subsequently incubated at room temperature for 1.5 hrs on a horizontal orbital microplate shaker at 450rpm. Following this incubation, solution from all wells was decanted and wells were washed 3 times with wash buffer (300µl). Detection antibody (100µl) was added subsequent to washing and the plate was incubated for 1 hr on a horizontal orbital microplate shaker at room temperature at 450rpm. Detection antibody was decanted and wells were again washed 3 times with 300µl of wash buffer before the addition of 100µl of enzyme solution and incubation for 30 mins on a horizontal orbital microplate shaker at 450rpm. Enzyme solution was decanted, wells were washed 3 times with wash buffer (300µl) and 100µl of substrate solution was added. The plate was incubated on a horizontal orbital microplate shaker at 450rpm until a blue colour was formed in all wells with increasing intensity in the standards according to the amount of GLP-1 present in the well. Stop solution (100µl) was subsequently added and the plate was shook gently by hand before absorbance was read at 450nm and 590nm using a FlexStation 3. A typical GLP-1 standard curve is shown with absorbance versus log GLP-1 concentrations in Fig. 2.3.

2.3.3: Measurement of GIP

Rat/mouse GIP total ELISA kits were used to quantify the GIP content of both plasma and cell culture supernatant samples. This is a sandwich based ELISA which measures both the 1-42 and 3-42 forms of GIP. For cell supernatant samples, 10µl of supernatant collected in section 2.1 was used while 10µl of plasma was used for plasma samples. Each kit contained one 96 well plate.

Components of the ELISA were pre-warmed to room temperature before commencing the assay as per manufacturer's protocol. A 2000pM GIP standard underwent a series of dilutions in order to obtain a standard curve ranging from 8.2 to 2000pM. Quality control 1 and quality control 2 were reconstituted in µQ H₂O with excess kept at -20°C. Before addition of any samples to the plate, each well was incubated for 5 mins in 300µl of wash buffer. Following this incubation, wash buffer was decanted and standard and quality control wells received 80µl of assay buffer and 10µl of matrix solution. Blank wells received 90µl of assay buffer and 10µl of matrix solution. Unknown sample wells received 90µl of assay buffer. Blank, standard and quality control wells were tested in duplicate while unknown samples were assayed in triplicate. Following plate preparation, 10µl of standards, quality controls and unknown samples were added to the appropriate wells. The plate was subsequently incubated at room temperature for 1.5 hrs on a horizontal orbital microplate shaker at 450rpm. Following this incubation, solution from all wells was decanted and wells were washed 3 times with wash buffer (300µl). Detection antibody (100µl) was added subsequent to washing and the plate was incubated for 1 hr on a horizontal orbital microplate shaker at room temperature at 450rpm. Detection antibody was decanted and wells were again washed 3 times with 300µl of wash buffer before the addition of 100µl of enzyme solution and incubation for 30 mins on a horizontal orbital microplate shaker at 450rpm. Enzyme solution was decanted, wells were washed 3 times with wash buffer (300µl) and 100µl of substrate solution was added. The plate was incubated on a horizontal orbital microplate shaker at 450rpm until a blue colour was formed in all wells with increasing intensity in the standards according to the amount of GIP present in the well. Stop solution (100µl) was subsequently added and the plate was shook gently by hand before absorbance was read at 450nm and 590nm using a FlexStation 3. A typical GIP standard curve is shown with absorbance versus log GIP concentrations in Fig. 2.4.

2.4: Lactate dehydrogenase (LDH) assay

2.4.1: Materials

CytoTox96 non-radioactive cytotoxicity assay kits were purchased from Promega (Madison, USA). Phosphate buffered saline (PBS) tablets were sourced from Analab (Dublin, Ireland).

2.4.2: Measurement of LDH activity

Cell supernatants were collected as outlined in section 2.1 for the measurement of LDH. Cellular release of LDH was used as an indicator of membrane integrity following the addition of test solutions to cells. This assay is based on the principle that the breakdown of the cell membrane will result in the presence of LDH. The presence of LDH will convert the tetrazolium salt (INT) into a red formazan product with the amount of colour produced being directly proportional to the amount of LDH present. The assay was performed as per manufacturer's instructions. All reagents were thawed and incubated at room temperature. Substrate mix was reconstituted using 12ml of assay buffer. The cell supernatant (50µl) and substrate reagent (50µl) were added to each well of a 96 well plate. The plate was covered and protected from light for 30 mins at room temperature. To stop the reaction, 50 µl of stop solution was added to all wells. Absorbance was measured at 490 nm using a Flexstation 3. Cellular LDH release (% of control) was quantified using the following equation:

$$\text{Cellular LDH release} = 100 \times \frac{\text{Experimental LDH release (OD, 490nm)}}{\text{Control LDH release (OD, 490nm)}}$$

2.5: Pancreatic islet isolation

2.5.1: Materials

Collagenase-P derived from *Clostridium histolyticum* was obtained from Sigma-Aldrich (Poole, Dorset, UK). All other chemicals used were obtained from BHD chemicals as outlined previously.

2.5.2: Isolation of mouse pancreatic islets

KRBB isolation solution was prepared as described in section 2.1.6 except for $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaHCO_3 and HEPES which were used at concentrations of 2.56mM, 20mM and 16mM respectively. Isolation solution (5ml) without KRBB was used to prepare collagenase (1.4mg/ml) which was kept on ice. Mouse pancreatic islets were isolated from lean and HFF Swiss TO mice (Envigo, Huntingdon, UK) by enzymatic collagenase digestion (Moskalewski 1969). Mice were culled using cervical dislocation after being anaesthetised by isoflurane. Pancreases were extracted and placed in a universal tube containing collagenase solution and placed on ice. This process was repeated for each pancreas which were then incubated at 37°C in a preheated shaking water bath for 5 mins at high speed (300 cycles/ min). After this incubation, samples were vigorously shaken

manually for 10 secs until the solution was homogenous. In order to ensure the breakdown of large sections of pancreas, tissues were further minced using scissors for 1 min. The pancreases then underwent another 5 min incubation at 37°C and were again shaken vigorously for 10 secs. To stop islet over digestion, ice cold isolation buffer was added to all tubes prior to centrifugation at 1,200rpm for 2 mins using a Beckman microcentrifuge (Beckman Instruments, UK). Following this step, the supernatant was discarded and the remaining pellet resuspended in 10ml of isolation solution. To remove any undigested pancreatic tissue the resuspended islet solution was poured through a tea strainer. The remaining solution centrifuged at 1,200rpm for 2 mins and the supernatant discarded. The subsequent pellet was resuspended in 25ml of RPMI-1640 and transferred to tissue culture dishes. Isolated islets were incubated at 37°C, 5% CO₂ and 95% air for 48 hrs to allow for the detachment of exocrine tissue.

2.5.3: Acute insulin secretion tests

Glucose solutions (1.1mM, 5.6mM and 16.7mM) were prepared in KRBB as described in section 2.1.6. Test solutions were prepared in 5.6mM and 16.7mM glucose in KRBB. Isolated islets were visualised using a phase contrast microscope (Zeiss, Germany) and transferred to a microcentrifuge tube (10 islets per tube) using a micropipette. All tubes were centrifuged at 900rpm for 5 mins using a Beckman microcentrifuge (Beckman Instruments, UK) and excess media discarded. Islets were subsequently incubated for 1 hr with 1.1mM glucose in KRBB (1ml) at 37°C, 5% CO₂ and 95% air before another centrifugation at 900rpm for 5 mins after which the supernatant was decanted. Each tube received 1ml of test solution as appropriate. Tubes were again incubated for 1hr at 37°C, 5% CO₂ and 95% air and centrifuged at 900rpm for 5 mins following this incubation. Supernatants (980µl) from all tubes were collected and transferred to labelled LP3 tubes (Sarstedt, Germany) before insulin measurement via RIA. The remaining pellets were treated with 1ml ice cold acid ethanol (1.5% HCl (v/v), 75% ethanol (v/v) and 23.5% H₂O (v/v)). Tubes underwent another centrifugation step at 900rpm for 5 mins before transfer to LP3 tubes for measurement of insulin content via RIA. From the 1ml of collected supernatant for insulin content, 20µl was diluted with 180µl of working RIA assay buffer for insulin RIA with. Insulin secretion from islets was measured using 200µl of islet supernatant. Insulin release (% of total pancreatic insulin content) was calculated using the following equation:

$$\text{Insulin Release (\% of content)} = 100 \times \frac{\text{Insulin release}}{\text{Insulin release} + \text{Total insulin content}}$$

2.6: Histology

2.6.1: Materials

Paraformaldehyde (PFA), 4' 6-diamidino-2-phenylindole (DAPI) nuclear stain, p-Phenylenediamine (antifade) and ethanol were purchased from Sigma-Aldrich (Poole, Dorset, UK). Xylene was obtained from VWR (Dublin, Ireland). All other chemicals used were obtained as described previously. Suppliers of all primary and secondary antibodies used for immunohistological staining are shown in Table 2.1.

2.6.2: Immunofluorescence staining of GLUTag, pGIPneo STC-1 and STC-1 cells

GLUTag, pGIPneo STC-1 and STC-1 cells were harvested as described in section 2.1. Cells were seeded for 48 hrs at a density of 40,000 cells per drop on polylysine-coated slides (25 x 75 x 1mm, VWR) with 3 drops per slide. Cells were incubated at 37°C, 5% CO₂ and 95% air and in tissue culture dishes with 15ml of DMEM culture media. Following incubation, slides were washed twice in PBS for 5 mins before cells fixation in 4% PFA/PBS (v/v) for 20 mins at room temperature. Fixed slides were washed in PBS 3 times for 5 mins. Antigen retrieval was performed by incubation in 50mM sodium citrate (pH 6.0) at 95°C for 30 mins. Prior to addition of the primary antibody, slides were blocked with 2% BSA (w/v) for 1 hr to prevent non-specific binding. Primary antibodies (200µl per slide) were diluted in blocking solution and added at appropriate concentrations as shown in Table 2.1, and slides were incubated overnight at 4°C or 1 hr at 37°C. Subsequent to primary antibody incubation, slides were washed in PBS 3 times for 5 minutes with appropriate secondary antibodies (200µl per slide) being diluted in blocking solution and added for 45 mins at 37°C, protected from light. Following secondary antibody incubation, slides were washed 3 times in PBS for 5 mins and DAPI (0.1µg/ml, 200µl per slide) was added to slides which were incubated at 37°C for 30 mins. Finally slides were washed twice in PBS for 5 mins and mounted with antifade before fixation with clear nail varnish. Slides were analysed under the 350nm filter (DAPI), fluorescein isothiocyanate (FITC) filter (488nm) and tetramethylrhodamine isothiocyanate filter (TRITC) filter (594nm) using a fluorescent microscope (Olympus BX51 microscope, South-on-Sea, UK) and images acquired using the DP70 camera.

2.6.3: Tissue processing and immunofluorescence staining of tissue

Non-fasted mice were culled by cervical dislocation after anaesthetisation by isoflurane. Pancreases were extracted and cut in half from head to tail while small intestinal tissue was arranged into the

“Swiss Roll” technique as described previously (Moolenbeek, Ruitenberg 1981). Extracted tissue was placed into labelled cassettes before fixation in 4% PFA (w/v) for at least 48 hr at 4°C. Samples were embedded in paraffin wax using an automated Leica TP1020 automated tissue processor (Leica Bio systems, Germany) as per manufacturer’s guidelines. Briefly, tissues in labelled cassettes were placed into the tissue basket and attached placed into the tissue basket holder. The tissue basket holder revolved around the instrument transferring cassettes between 4% PFA (w/v), increasing concentrations of ethanol, xylene and molten paraffin wax as follows: 70%, 80%, 96% ethanol (v/v) for 2 hr each, 100% ethanol 2 changes for 2 hr, xylene 2 changes for 1 hr 30 mins and 2 changes in paraffin wax for 4 hr. Subsequent to this processing, tissues were placed into plastic moulds, appropriately orientated, covered in molten paraffin wax and labelled cassettes attached to the top. Molten paraffin wax blocks were allowed to set for 24 hrs at room temperature, after which blocks were removed from the moulds and stored at 4°C.

A microtome (RM2035, Leica, Germany) was used to cut 8µm tissue sections, which were subsequently floated on a 37°C water bath and lifted onto polylysine-coated slide (25 x 75 x 1mm, VWR). To ensure no bias in tissue selection, a random number generator was used to select which tissue sections were collected. Slides were dried on a hot plate at 37°C overnight. Slides with paraffin embedded tissue were dewaxed twice in xylene for 10 mins. Tissue was rehydrated using decreasing concentrations of ethanol as follows: 100% ethanol twice for 5 mins, 95% ethanol (v/v) for 5 mins, 80% ethanol (v/v) for 5 mins and distilled water for 5 mins. Antigen retrieval, blocking, staining procedure, mounting and visualisation of slides was performed as outlined above (Section 2.6.2).

2.7: Animal models

All animal experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and the ARRIVE guidelines for experiments involving animals (Kilkenny *et al.* 2012). All mice were housed in an air-conditioned room maintained at 22 ± 2°C with a 12 hr dark: 12 hr light cycle (08:00-20:00).

2.7.1: Swiss TO mice

Male Swiss TO mice (8 wk) were purchased from Envigo (Huntingdon, UK) derived from a colony originally sourced from Statens Serum Institute (Copenhagen, DK). Mice were housed in single cages and supplied drinking water and standard rodent maintenance diet (60% carbohydrate, 30% protein, 10% fat (12.99 kJ/g), Trouw Nutrition, Cheshire, UK) *ad libitum*. Swiss TO mice on a

standard rodent maintenance diet exhibited normal glycaemic levels, no weight gain and had normal glucose tolerance.

2.7.2: Diet induced diabetic mice

Male NIH Swiss mice (8-10 wks) were obtained from Harlan (Blackthorne, UK) and derived from a colony originally sourced from the National Institute of Health (Maryland, USA). Mice were individually housed and supplied with drinking water and high fat diet (35% carbohydrate, 20% protein, 45% fat (26.15 kJ/g), Special Diet Service, Essex, UK) *ad libitum* for 5 months to induce obesity-diabetes. High fat fed (HFF) mice had increased body weight and hyperglycaemia and also exhibited impaired glucose tolerance when compared to NIH Swiss mice on the standard rodent maintenance diet as described previously (Bailey *et al.* 1986). This model of diet-induced diabetes in mice simulates a high fat western diet and induces several important clinical features identified in human obesity.

2.7.3: Multiple low dose streptozotocin induced-diabetic mice

Age-matched (26 wks), male Swiss TO mice were sourced from Envigo (Huntingdon, UK). Mice were housed in single cages and were supplied drinking water and standard rodent maintenance diet (60% carbohydrate, 30% protein, 10% fat (12.99 kJ/g), Trouw Nutrition, Cheshire, UK) *ad libitum*. Streptozotocin is a glucose analogue which is cytotoxic to the insulin secreting β -cells of the pancreas (Szkudelski 2001). Due to the similarities in structure with glucose, streptozotocin is able to enter β -cells through the GLUT2 receptor and induce diabetes through β -cell toxicity and a T-cell mediated immune response against β -cells in the pancreas (Wang, Gleichmann 1998, Szkudelski 2001). The alkylating properties of streptozotocin mean that free radicals are added to DNA present in β -cells and destroying the cell and preventing it from metabolising glucose due to the breakdown of mitochondrial DNA (Szkudelski 2001). The effects of multiple low dose streptozotocin induced diabetes on rats has previously shown to result in mild hyperglycaemia, hypoinsulinaemia, hyperphagia and polydipsia with no adverse effects on weight gain (Kobayashi, Olefsky 1979). Diabetes was chemically induced in 4 hr fasted Swiss TO mice through 4 intraperitoneal injections of streptozotocin (40mg/kg body weight, 0.1M sodium citrate, pH 4.5) which were received on alternate days. Diabetes was confirmed through an oral glucose tolerance test (OGTT) which was performed 14 days after the first injection of streptozotocin injection (day 0) on 18 hr fasted Swiss TO mice.

2.7.4: Acute food intake in 3 hr trained mice

Age matched male Swiss TO mice (20-24 wk) were sourced from Envigo (Huntingdon, UK). Mice were housed in single cages and were supplied drinking water *ad libitum* and standard rodent maintenance diet (60% carbohydrate, 30% protein, 10% fat (12.99 kJ/g), Trouw Nutrition, Cheshire, UK). Mice were habituated to feeding for 3 hr per day as described previously (O'Harte *et al.* 1998a). Briefly, mice were initially supplied standard rodent diet *ad libitum*. After 7 days this was reduced to 12 hrs for another 7 days. There was a further reduction to 6 hr access to diet for 7 days before a final reduction to 3 hrs. Mice continued on 3 hr access to standard rodent maintenance diet for 7 days before experimentation. All GPCR agonists were administered in 0.9% saline (n=8).

2.8: Acute in-vivo glucose tolerance tests

2.8.1: Materials

Fluoride/ heparin coated microcentrifuge tubes were purchased from Sarstedt (DE). D-glucose and NaCl were obtained from BDH Chemicals (Poole, Dorset, UK). Bayer Contour Next glucose meter and strips were sourced from Williams Medical Supplies (Rhymney, UK).

2.8.2: Glucose tolerance tests

Acute oral glucose tolerance tests were carried out in Swiss TO mice on standard rodent maintenance diet. Mice were fasted for 18 hrs prior to testing. Groups of mice (n=6) were maintained as described in section 2.7.1 with food withheld for the duration of the experiment. Blood samples were collected via tail vein bleeding of conscious mice prior to the commencement of testing (0 mins). GPCR agonists were administered orally in combination with glucose (18mmol/kg BW) or in combination with glucose and the DDP IV inhibitor Sitagliptin Phosphate (50mg/kg BW). Blood was collected at 15, 30, 60, 90 and 120 mins post administration. Collected blood was centrifuged at 13,000rpm for 5 mins at 4°C using a Beckman microcentrifuge (Beckman Instruments, UK). Plasma was aliquoted into microcentrifuge tubes and stored at -20°C prior to glucose, insulin, GLP-1 and GIP measurement.

2.8.3: Biochemical analysis

Blood glucose determination was performed using a Bayer Contour Next meter (Leverkusen, DE). This is an automated glucose analyser that can read glucose concentrations up to 35mM. Plasma

insulin was quantified using insulin RIA as previously described in section 2.2. Plasma (20µl) was diluted (1:10) in working assay buffer and results multiplied accordingly. Plasma GLP-1 and GIP were measured using ELISA as described in section 2.3. For GLP-1 samples, plasma was diluted 1:2.5 and all results were multiplied accordingly.

2.9: Chronic biological effects of GPCR agonist administration both as a monotherapy and combination therapy in multiple-low streptozotocin induced-diabetic mice

2.9.1: Materials

Abn-CBD and AS-1269594 were purchased from Tocris Bioscience (Bristol, UK). Sitagliptin Phosphate was sourced from Apex Bioscience (Durham, North Carolina, USA). Multi species GLP-1 total and rat/mouse GIP total ELISA kits were supplied by Merck Millipore (Watford, UK). Streptozotocin and bovine insulin were purchased from Sigma-Aldrich (Poole, Dorset, UK). D-glucose and NaCl were obtained from BDH Chemicals (Poole, Dorset, UK). Materials for triglyceride assay were purchased from Instrumentation Laboratory (Warrington, UK) while kits for total cholesterol and HDL were purchased from Randox (Co Antrim, UK).

2.9.2: Treatment procedure and parameters assessed

The long term effects of oral Abn-CBD and AS-1265974 (0.1µmol/kg BW) or saline (0.9% w/v NaCl) in multiple low dose streptozotocin-induced diabetic Swiss TO mice (n=6) (section 2.7.3) was assessed over a period of 21 days. Hyperglycaemia was confirmed in diabetic mice through an oral glucose tolerance test which was performed 14 days after the first streptozotocin injection (day 0) on 18 hr fasted NIH Swiss mice as previously described (section 2.8.2). Fluid intake, food intake, body weight, non-fasted blood glucose, insulin, GLP-1 and GIP concentrations were monitored every 3 days. Blood was collected via tail vein bleeding of conscious mice and was collected and analysed for blood glucose, insulin, GLP-1 and GIP as described in section 2.8.4. After the testing period, glucose tolerance tests (18mmol/kg body weight) were carried out to assess glucose tolerance on 18 hr fasted streptozotocin treated mice and Swiss TO mice on standard rodent maintenance diet as outlined in section 2.8.2.

2.9.3: Insulin sensitivity

Prior to the injection of insulin, blood glucose was measured in non-fasted Swiss TO mice via tail vein bleeding using the Bayer Contour glucose meter. Bovine insulin (25U/kg) in 0.9% saline was administered using intraperitoneal injection of bovine insulin (25U/kg) and blood glucose was monitored at 30 and 60 mins post injection.

2.9.4: Dual energy X-ray absorption (DEXA)

Non-fasted Swiss TO mice were culled by cervical dislocation after being anaesthetised using isoflurane, and subsequently arranged on the specimen tray to ensure both limbs and tail were extended away from the body. In order to ensure calibration and quality control, 25 measures of the aluminium/lucite phantom (0.069g/cm², 12.0% fat) were taken using a Lunar PIXImus Dual energy X-ray absorption (DEXA) system (software version 1.4x) before any specimens were measured. DEXA scanning was performed on all carcasses as per manufacturer's guidelines. The Lunar PIXImus DEXA system measures percentage body fat as well as bone mineral density (BMD, g/cm³) and bone mineral content (BMC, g) using a fully integrated densitometer. A representative DEXA scan of a lean Swiss TO mouse and a multiple low dose streptozotocin-induced diabetic mouse are shown in Fig. 2.5.

2.9.5: Measurement of plasma hormones

Blood was collected via tail vein bleed on conscious mice every 3 days for biochemical analysis on a variety of hormones. Blood glucose determination was performed using a Bayer Contour Next meter (Leverkusen, DE) every 3 days. Plasma insulin was quantified using insulin RIA as previously described in section 2.2. Rat/mouse GIP total ELISA kit (Millipore, Watford, UK) was used to measure total GIP concentrations in mouse plasma following chronic treatment. Multi species GLP-1 total ELISA was used to measure total GLP-1 content of mouse plasma. These sandwich based ELISAs are described in detail in section 2.3.

2.9.6: Assessment of plasma lipid profiles

Plasma triglyceride and HDL cholesterol concentrations were measured using an I-lab 650 clinical chemistry instrument (Instrumentation Laboratory, Warrington, UK).

2.10: Determination of mRNA expression in BRIN-BD11, GLUTag and STC-1 cells and NIH Swiss mouse pancreas and small intestine

2.10.1: Materials

TRIzol reagent, propan-2-ol and chloroform was supplied by Sigma (Poole, Dorset, UK). Superscript II reverse transcriptase RNase H kit was supplied by Invitrogen (Paisley, UK). Eppendorf real-time PCR tube strips & masterclear cap strips were sourced from Bio-Rad Laboratories (Hertfordshire, UK). LightCycler® 480 SYBR Green I Master was obtained from Roche (Basel, CH).

2.10.2: mRNA extraction and conversion to cDNA

mRNA was extracted from all cell lines and tissue using the phenol chloroform method as described previously (Chomczynski, Sacchi 1987). Briefly, cells and tissue were harvested as previously mentioned (Sections 2.1. and 2.6.3). Tissue was homogenised lysed in an LP3 tube with 1ml of TRIzol reagent. Cells were seeded in 6-well plates, treated with test solutions for the appropriate time and lysed using TRIzol on ice for 10 mins. Cells were agitated with gentle stirring. Tissue and cells were transferred to a fresh microcentrifuge tube and 200µl of chloroform was added. This suspension was agitated and incubated at room temperature for 10 mins before undergoing centrifugation at 12000g for 15 mins at 4°C (MIKRO 200R, Hettich Zentrifugen, Germany). The clear upper aqueous phase containing mRNA was transferred to a new microcentrifuge tube subsequent to centrifugation and 0.5ml of propan-2-ol was added. The interphase and organic phase was discarded. The mixture was agitated and incubated at room temperature for 10 mins before centrifugation at 12000g for 10 mins at 4°C. The supernatant was discarded and 70 % ethanol was used to wash the pellet of mRNA off the side of the microcentrifuge tube and centrifuged at 12000g for 10 mins at 4°C. This was repeated for a total of 3 times. Subsequent to 3 washes of 70% ethanol, the supernatant was discarded and pellets air dried at room temperature for 10 mins. Following air drying, the pellet was suspended in 30µl of RNase free water and boiled at 70°C for 5 mins before determination of the mRNA yield using a nanophotometer (Implen, version 2.0). The quality of mRNA was determined by 260/280 ratios with ratios between 1.9 and 2.1 being deemed acceptable. The 260/230 ratios were also measured with the requirement that this value was higher than the 260/280 ratio and greater than 2. From this mRNA, 1-5 µg was converted to cDNA using the superscript II reverse transcriptase – RNase H kit as per suppliers' instructions. mRNA samples were stored long term at -80°C while cDNA was stored at -20 °C.

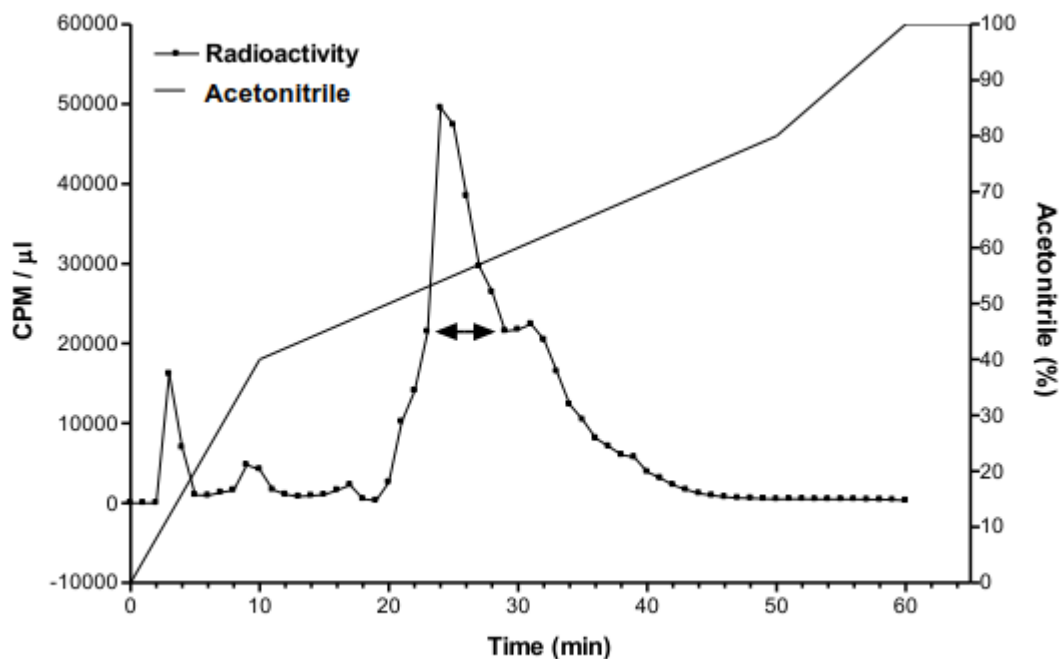
2.10.3: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH. Primer sequences for qPCR are listed in Table 2.2

2.11: Statistics

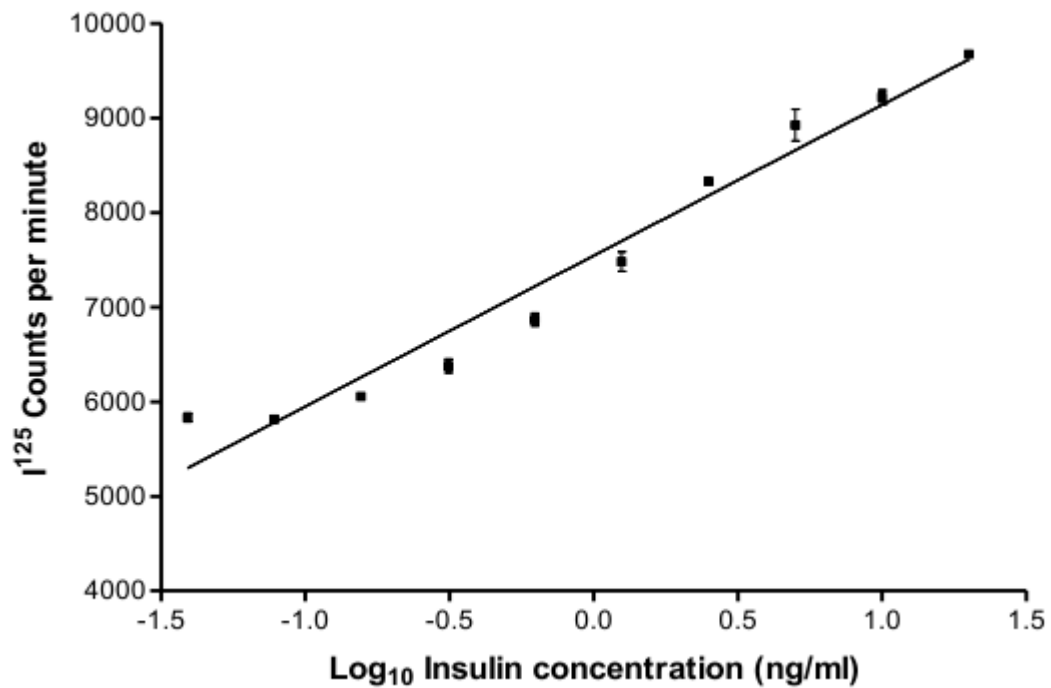
Data are expressed as the mean \pm the standard error of the mean (SEM). Results were compared using the Student's t-test on Prism graph pad version 5.0. Incremental area under the curve (AUC) with baseline subtraction, were calculated using Prism graph pad. Differences in data were considered to be statistically significant for $p < 0.05$.

Figure 2.1: Typical HPLC profile representing iodinated bovine insulin



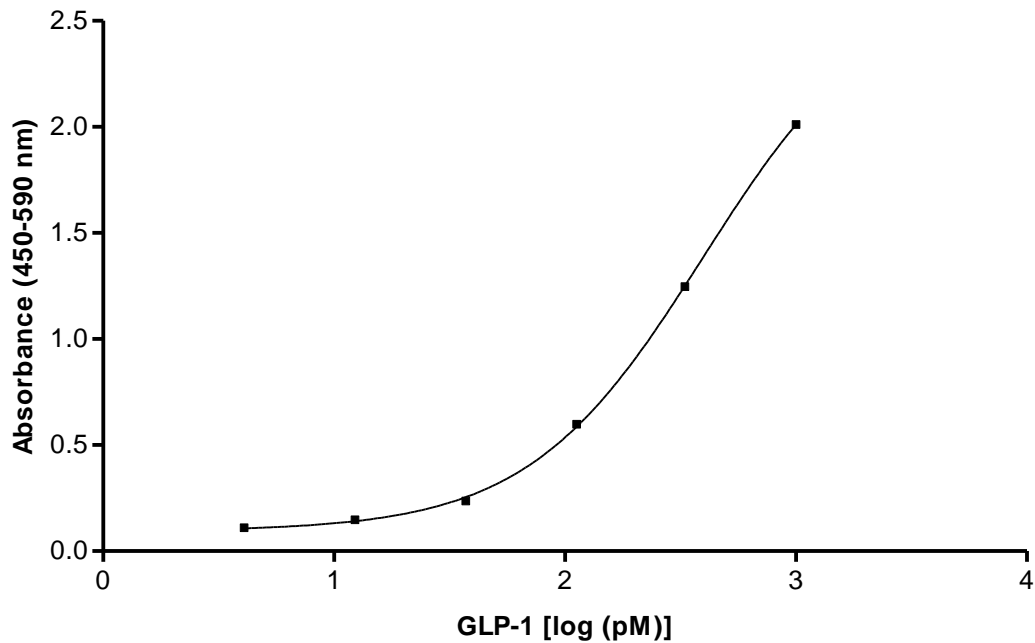
Bovine insulin labelled with Na^{125}I was prepared using the iodogen method described in section 2.2.2. Separation of bound and unbound fractions was achieved by RP-HPLC using a Vydac C-8 column. Elution of the iodination reaction mixture occurred using 70% acetonitrile/ H_2O (v/v) increased to 40% after 10 min, to 80% over 40 mins and 80- 100% over 10 min. Unbound Na^{125}I was initially eluted between 3-6 mins while bovine insulin labelled with Na^{125}I was eluted between 24-30 mins as indicated by the black arrows on the graph. Fractions with the highest CPM underwent antibody binding testing and fractions with similar binding were pooled and stored at 4°C.

Figure 2.2: Typical standard curve obtained for insulin RIA from rat insulin standards



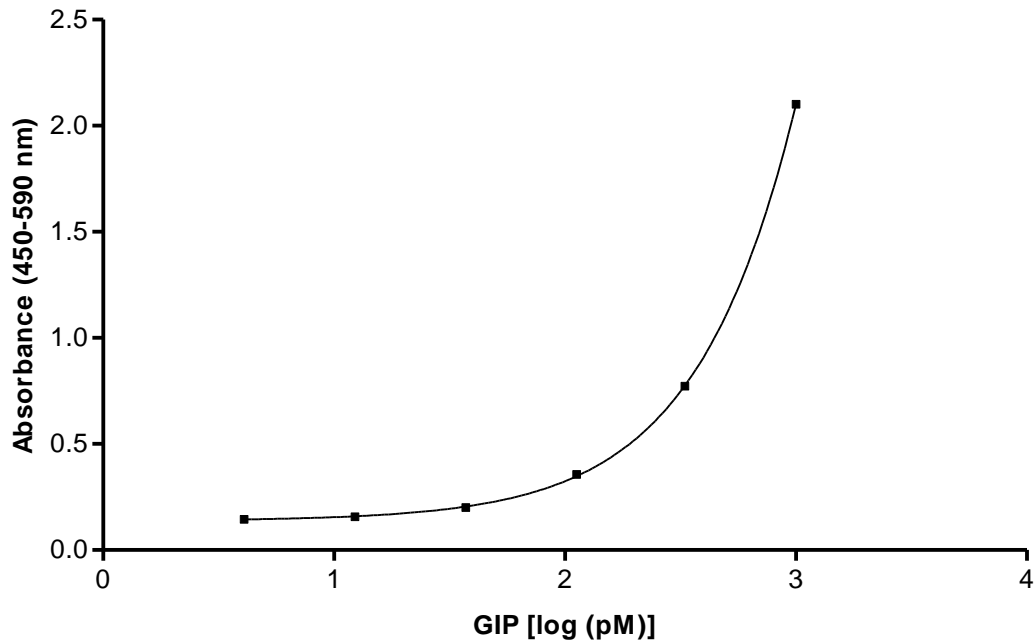
A rat insulin standard curve ranging from 0.039-20ng/ml by serial dilutions of the stock standard ($R^2 = 0.96$). Unknown values were interpolated from the standard curve and insulin concentrations anti-logged into ng/ml. Values are \pm SEM (n=3)

Figure 2.3: Typical standard curve obtained for multi species GLP-1 total ELISA



A GLP-1 standard curve with standard concentrations of 4.1-1000pM by serial dilution of a 1000pM stock ($R^2 = 0.9990$). Absorbance of unknown values was used to interpolate their concentration from the standard curve to quantify GLP-1 concentration. Quality control samples (QC1, QC2) which both fell within the recommended range were provided by the supplier and used to validate the assay. Values are \pm SEM (n=2).

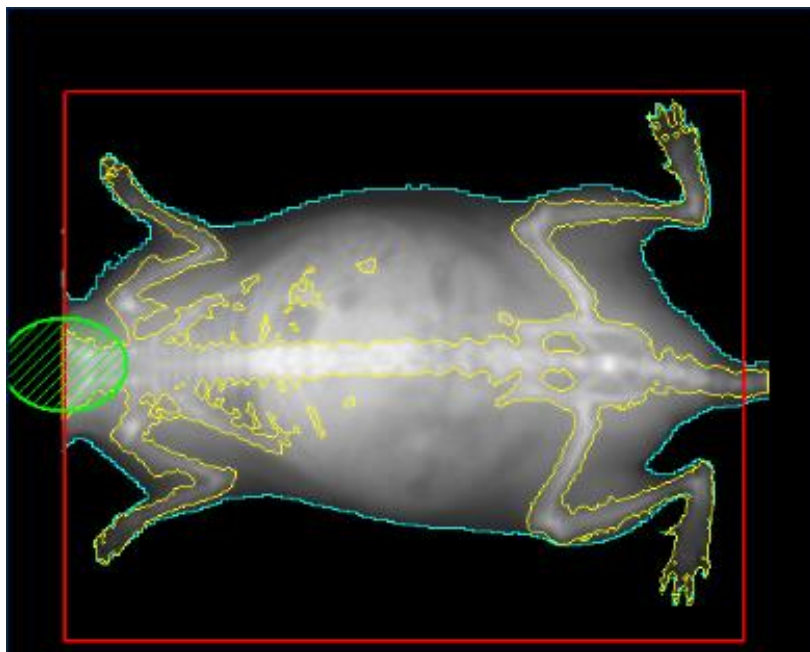
Figure 2.4: Typical standard curve obtained for rat/mouse GIP total ELISA



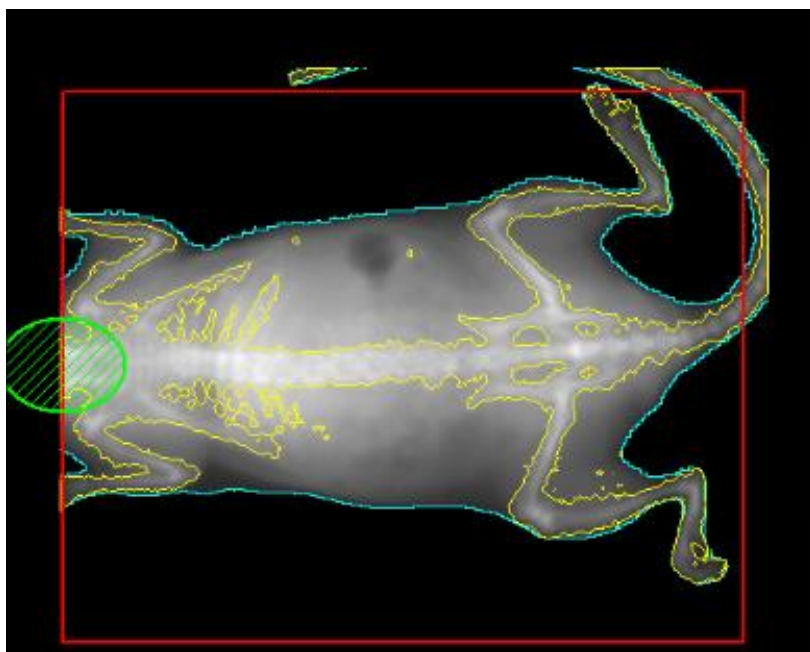
A GIP standard curve with standard concentrations of 8.2-2000pM by serial dilution of a 2000pM stock ($R^2 = 0.9990$). Absorbance of unknown values was used to interpolate their concentration from the standard curve to quantify GIP concentration. Quality control samples (QC1, QC2) which both fell within the recommended range were provided by the supplier and used to validate the assay. Values are \pm SEM (n=2).

Figure 2.5: Representative Dual energy X-ray absorption (DEXA) images of (A) a lean Swiss TO mouse and (B) a multiple low dose streptozotocin-induced diabetic Swiss TO mouse.

A.



B.



Mice were analysed after prior calibration and quality control with the aluminium/lucite phantom (0.069 g/cm², 12.0% fat) obtained by DEXA using a PIXImus system (software version 1.4x).

Table 2.1

Primary Antibody	Host Species	Dilution Used	Supplier
<i>Insulin</i>	Guinea-pig	1:500	Abcam (Cambridge, UK)
<i>Glucagon</i>	Mouse	1:100	Abcam (Cambridge, UK)
<i>GLP-1</i>	Goat	1:40	Santa Cruz Biotechnology (CA, USA)
<i>GIP</i>	Goat	1:40	Santa Cruz Biotechnology (CA, USA)
<i>PYY</i>	Goat	1:40	Santa Cruz Biotechnology (CA, USA)
<i>GPR39</i>	Rabbit	1:1000	Abcam (Cambridge, UK)
<i>GPR55</i>	Rabbit	1:150	Cayman Chemical (Michigan, USA)
<i>GPR75</i>	Rabbit	1:50	Santa Cruz Biotechnology (CA, USA)
<i>GPR119</i>	Rabbit	1:50	Santa Cruz Biotechnology (CA, USA)
<i>GPR120</i>	Rabbit	1:50	Santa Cruz Biotechnology (CA, USA)
Secondary Antibody			
<i>Anti-guinea pig alexa fluor 488nm</i>	Mouse	1:400	Molecular probes, Life Technologies (Paisley, UK)
<i>Anti-mouse alexa fluor 488nm</i>	Goat	1:400	Molecular probes, Life Technologies (Paisley, UK)
<i>Anti-goat alexa fluor 488nm</i>	Donkey	1:400	Molecular probes, Life Technologies (Paisley, UK)
<i>Anti-rabbit alexa fluor 594nm</i>	Goat	1:400	Molecular probes, Life Technologies (Paisley, UK)

Primary and secondary antibodies used for immunocytochemistry and immunohistochemistry throughout this thesis. Antibodies were optimised using serial dilutions as per the manufacturer's datasheet.

Table 2.2

Gene targeted		Primer sequence	Genome
GPR39	Forward	CCTCACCCGGAGTGACTATC	Mouse
	Reverse	GGCGCAACACTTTTCTGTCA	
GPR55	Forward	ATTTGGAGCAGAGGCACGAA	Mouse
	Reverse	AGGTTGAGAACCAGGCCAAG	
GPR75	Forward	AGCTGGTATCAGCGGTCAAC	Rat
	Reverse	CGACAACACGATGACCATGC	
GPR75	Forward	TCACCTGTGTGGTCATCGTG	Mouse
	Reverse	GAAAGCCCAGGCCAGTGTAT	
GPR119	Forward	GCCTTCGGATGGCATTGTGTC	Mouse
	Reverse	GTGGGAGGAAGCCGATAAGG	
GPR120	Forward	CTGGGGCTCATCTTTGTCGT	Mouse
	Reverse	ACGACGAGCACTAGAGGGAT	
Proglucagon	Forward	CAGAAGAAGTCGCCATTGCC	Mouse
	Reverse	GAAGTCCCTGGTGGCAAGAT	
PC1	Forward	TTCATAGGAGATATAAGCCCTTCC	Mouse
	Reverse	CCAAACGCAAAAGAAGGCGA	
GAPDH	Forward	GGACCTCATGGCCTACATGG	Mouse
	Reverse	TAGGGCCTCTCTTGCTCAGT	
GAPDH	Forward	AGTGCCAGCCTCGTCTCATA	Rat
	Reverse	GATGGTGATGGGTTTCCCGT	

Primer sequences for all genes that were analysed using qPCR.

Chapter 3

Activation of GPR75 increases insulin and incretin hormone secretion *in vitro* and *in vivo*

3.1: Overview of results

GPR75 is a G-protein coupled receptor which was originally found in the human eye. CCL5 is a proinflammatory chemokine that has been shown to activate GPR75 leading to increased insulin secretion from the pancreas. There are currently no studies which have studied the role of GPR75 activation on intestinal hormones and there is currently only one previously published study which identifies a role for GPR75 in glucose homeostasis. The current study aims to determine the role of the novel GPCR, GPR75, activation by CCL5 on intestinal and pancreatic function and glucose homeostasis. The cytotoxicity of CCL5 to β -cells was determined by MTT assay. Immunohistochemistry and qPCR was used to determine the expression of GPR75 in both lean and HFF NIH Swiss mouse pancreas and small intestine. Secretory studies were performed using the pancreatic BRIN-BD11 cell line. Immunocytochemistry was performed using BRIN-BD11, α TC1.9, STC-1 and GLUTag cells. The acute *in vivo* effects of CCL5 (25nmol/kg BW) were examined orally and via IP injection in fasted male Swiss TO mice (n=6). CCL5 (25nmol/kg BW) delivered orally was tested as a lone treatment and in combination therapy with Sitagliptin (50mg/kg BW, n=6).

In 5.6mM glucose CCL5 increased insulin secretion 1.3 to 1.8 fold (p0.05 to p<0.001) while in hyperglycaemic conditions (16.7mM glucose) insulin secretion was augmented 1.1 to 1.8-fold (p<0.01 to p<0.001). At both glucose conditions, CCL5 had no cytotoxic effects. qPCR was used to determine the expression of GPR75 in lean and HFF mouse pancreas and small intestine and this was confirmed with immunohistochemistry. A HFF diet led to a 36.7% (p<0.01) increase in pancreatic GPR75 expression (n=6) with GPR75 being co-localised with the β -cells of the pancreas but not α -cells. BRIN-BD11 cells treated for 20 mins with CCL5 resulted in increased insulin expression (p<0.001) with this being exhausted after 4hr treatment (p<0.05-p<0.01) in 5.6 and 16.7mM glucose when compared to vehicle control as determined by immunohistochemistry. GPR75 expression was increased after both 20 min (p<0.001) and 4hr treatment (p<0.05-p<0.01) in both 5.6 and 16.7mM glucose when compared to vehicle control.

In the intestine a 46.4% (p<0.05) reduction in GPR75 expression was observed in HFF mice compared to lean (n=6). The expression of GPR75 in the L and K-cells of the intestine was determined using double immunohistochemistry. GPR75 was confirmed in both the L and K-cells of the intestine as it was found to be co-localised of with GLP-1, GIP and PYY being observed. In GLUTag cells treated with CCL5 for 2 hrs both proglucagon (3.0-fold, p<0.001) and PC1 (2.3-fold, p<0.001) gene expression were increased compared to vehicle control.

IP injection of CCL5 (25nmol/kg BW) in fasted male Swiss TO mice (n=6) increased insulin secretion 1.4-fold (p<0.001) and resulted in a 1.3-fold reduction in blood glucose (p<0.001). When administered orally, CCL5 alone had the greatest reduction in blood glucose with a 1.9-fold

decrease being observed ($p < 0.001$). Insulin secretion was increased 1.4-fold ($p < 0.001$). While combination of CCL5 and Sitagliptin also reduced blood glucose, this was less than with CCL5 treatment alone as a 1.7-fold decrease resulting from this treatment ($p < 0.001$). The increase in insulin secretion was also lower than CCL5 administration alone with a 1.3-fold increase being observed ($p < 0.001$).

This study demonstrates that GPR75 is readily expressed in both the pancreas and intestine. This result suggests a role for GPR75 activation in both the pancreas and intestine can be utilised to help regulate glucose homeostasis. Therefore CCL5 activation of GPR75, and other future novel agonists, may be a potential therapy for Type 2 Diabetes. The identification of new GPR75 agonists and future research of GPR75 activation on intestinal endocrine cell function will help to elucidate the mechanisms through which this can be achieved. The *in vivo* results in this study show that CCL5 activates receptors found in the endocrine cells of the intestine.

3.2: Introduction

The human form of GPR75 was first identified in 1999, which found that a gene on chromosome 2p16 encoded a 543 amino acid long protein with a molecular weight of 59.4kDa (Tarttelin *et al.* 1999). GPR75 was originally only found to be present in the brain, eye, blood and spinal cord of humans, however as the mouse gene had yet to be cloned, the expression pattern of GPR75 in mice was not ascertained. Later research confirmed the presence of GPR75 in the mouse genome and showed it had 87% homology with human GPR75 (Visel, Thaller & Eichele 2004, Ignatov *et al.* 2006). Unlike human GPR75, mouse GPR75 is found on chromosome 11a4 (Ignatov *et al.* 2006). The receptor was most closely related to rat galanin receptor type 3 (25% homology), *C. elegans* neuropeptide Y receptor (24% homology) and porcine growth hormone secretagogue receptor type 1bAs (23% homology). As GPR75 was first identified to be present in the eye, research was carried out to try and identify mutations in GPR75 which may have an effect in age related macular degeneration (Sauer *et al.* 2001). A cohort of 535 patients affected by AMD discovered 6 pathogenic variants of GPR75 however no functional studies were carried out and further research is required to see if any of these may be a possible cause of AMD. Earlier research found no mutations in GPR75 in a screen of patient's who suffered from Doyme's honeycomb retinal dystrophy (Tarttelin *et al.* 1999) and further work into the effects of GPR75 on disorders in the eye need to be carried out.

The first identified GPR75 agonist was Chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES or regulated on activation, normal T cell expressed and *secreted*) which was shown to increase intracellular calcium, inositol trisphosphate and stimulation of the luciferase assay in cells which had been transfected with GPR75 (Ignatov *et al.* 2006). $G_{\alpha q}$ has previously been identified as

the G-protein α subunit which activates phospholipase C (PLC) activity (Maurice *et al.* 1993) and the presence of U73122, a PLC inhibitor, prevented CCL5 increasing intracellular calcium thus demonstrating that GPR75 signals through the $G_{\alpha q}$ pathway. Another cytokine, MIP1 α , was shown to stimulate luciferase gene expression, however no effect on intracellular calcium was observed and further work needs to be carried out to elucidate whether MIP1 α is an agonist for GPR75. CCL5 is produced endogenously by fibroblasts, platelets, macrophages, CD8⁺ T cells, endometrial cells and epithelial cells (Appay, Rowland-Jones 2001, Levy 2009) and has previously been shown to reduce HIV infection as it competes with HIV to bind to C-C chemokine receptor type 5 (CCR5), while having an effect in experimental autoimmune uveitis (Crane *et al.* 2001) as well as having proinflammatory effects (Alkhatib *et al.* 1996, Benamar, Geller & Adler 2008). These proinflammatory effects include the recruitment of T cells, monocytes, eosinophils, natural killer cells, basophils, mast cells and dendritic cells to sites of inflammation (Schall 1991, Appay, Rowland-Jones 2001). Only recently has a second GPR75 agonist been identified. 20-Hydroxyeicosatetraenoic acid (20-HETE) was found to bind to GPR75 in human cells with the same increase in inositol trisphosphate as seen with CCL5 treatment (Garcia *et al.* 2017) thus confirming the signalling of GPR75 through $G_{\alpha q}$. As of yet no antagonists for GPR75 have been identified.

The search for further GPR75 agonists and antagonists is important for several reasons. Firstly, CCL5 is able to signal through several receptors such as CCR1 (Neote *et al.* 1993), CCR3 (Daugherty *et al.* 1996), CCR4 (Power *et al.* 1995) and CCR5 (Samson *et al.* 1996), all of which are also GPCRs like GPR75 with which they have 12-16% sequence homology (Liu *et al.* 2013). Therefore it is important to determine the selectivity of CCL5 for the receptor being studied. This becomes less challenging when one CCL5 binding receptor is highly expressed while the others are seen at low levels, with such an expression pattern being demonstrated in the pancreas (Liu *et al.* 2013). Secondly, the activation of these chemokine receptors has been linked to a variety of syndromes (D'Ambrosio, Panina-Bordignon & Sinigaglia 2003) making it harder to elucidate the effects of GPR75 activation. CCL5 activity can be reduced through wortmannin which inhibits PI 3-kinase activity (Turner, Ward & Westwick 1995) although this effect has yet to be seen for GPR75.

The ability of GPR75 to increase PLC and thus intracellular calcium through the $G_{\alpha q}$ pathway led to an interest in the effects of GPR75 activation on insulin secretion (Liu *et al.* 2013). This research confirmed the presence of both CCL5 and GPR75 in both mouse and human pancreatic islets using immunohistochemistry and PCR. Immunohistochemistry and qPCR showed that the cytokine receptors GPR75 agonist CCL5 activate, namely CCR1, CCR3 and CCR5, were either massively downregulated or not present in both human and mouse pancreatic islets. Downregulation of GPR75 using siRNA in MIN6 cells demonstrated the specificity of CCL5 for GPR75 in the β cells of the pancreas while also confirming the activation of the $G_{\alpha q}$ pathway which was shown to be activated

in earlier research (Ignatov *et al.* 2006). The insulinotropic effect demonstrated in MIN6 cells was further shown in isolated mouse and human islets. CCL5 activation of GPR75 also resulted in increased insulin secretion *in vivo*. These results demonstrate a role for GPR75 in glucose homeostasis and it may be a potential therapy in the treatment of Type 2 Diabetes. There are high expression levels of CCR5 on T and B cells which can be activated by CCL5. NOD mice treated with a neutralising anti-CCR5 antibody had reduced β -cell destruction thus demonstrating a role for CCL5 in the autoimmune destruction of β -cells which is seen in Type 1 Diabetes. Therefore further research will need to be carried out to further determine the suitability of GPR75 activation for individuals with Type 1 Diabetes.

Previous work demonstrating the effect of GPR75 activation on insulin secretion (Liu *et al.* 2013) was carried out in pancreatic cells while the method of drug delivery *in vivo* bypassed the intestine. Interestingly CCL5 is cleaved by DPP-IV in the intestine (Proost *et al.* 1998). This study will elucidate the effects of GPR75 agonist CCL5 on the intestine *in vivo* as well as determine the expression pattern of GPR75 in a range of intestinal and pancreatic cell lines. The effects of activation of GPR75 on intestinal and pancreatic hormone secretion *in vivo* will also be determined.

3.3: Materials and methods

3.3.1: Materials

CCL5 was purchased from SynPeptide (SHA, CN). All other materials were sourced as described in Chapter 2.

3.3.2: Acute insulin secretion from pancreatic BRIN-BD11 cells

Cells were seeded in 24-well plates with 150000 BRIN-BD11 cells per well and incubated overnight in 1ml of RPMI 1640 media at 37°C and 5% CO₂. Following this incubation, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁵M concentrations of CCL5 with 10mM alanine as a positive control (n=8). After incubations, supernatants (950 μ l) were collected and frozen at -20°C until radioimmunoassay was carried out.

3.3.3: Acute effects of CCL5 on cell toxicity

Cells were seeded in 96-well plates with 40000 cells per well of BRIN-BD11 cells and incubated overnight in cell culture media (as described in Chapter 2, section 2.1.2) in an atmosphere of 5% CO₂ at 37°C. After 20hrs, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁵M concentrations of CCL5 with 1mM H₂O₂ as a cytotoxic control (n=3). After incubation, supernatant was decanted, cells were washed with HBSS and 0.5mg/ml MTT was added for 2hrs. Following MTT incubation, supernatant was discarded and DMSO was added to solubilise crystals. Optical density was subsequently measured at 570nm and 630nm using the Flexstation 3 (Molecular Devices, CA, USA).

3.3.4: Acute insulin secretion from isolated islets

Pancreatic islets were isolated from both lean and HFF Swiss TO mice by enzymatic collagenase digestion as outlined in Chapter 2, section 2.5.2. CCL5 and alanine (10mM) were added to KRBB buffer in 11.1mM glucose and acute insulin secretion assessed in isolated islets as previously described in Chapter 2, section 2.5.3.

3.3.5: Immunofluorescence staining in GLUTag, STC-1 cells and lean and HFF NIH Swiss mouse small intestine

GLUTag, pGIPneo STC-1 and STC-1 cells were seeded at a density of 40000 cells on to polylysine coated slides overnight at 37°C in an atmosphere of 95% air and 5% CO₂ as described in Chapter 2, section 2.1. Swiss TO mice were anaesthetised by isoflurane and killed by cervical dislocation. Pancreas and intestine was excised as described in Chapter 2, section 2.6.3. Immunohistochemistry was carried out by incubating with rabbit polyclonal anti-GPR75 and guinea pig polyclonal anti-insulin, mouse polyclonal anti-glucagon, goat polyclonal anti-GLP-1, goat polyclonal anti-GIP or goat polyclonal anti-PYY with the dilutions outlined in Chapter 2, Table 2.2.

3.3.6: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Chapter 2, Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The

reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH.

3.3.7: Acute *in vivo* glucose tolerance tests

Male Swiss TO mice were housed as described in Chapter 2, section 2.7. The effects GPR75 agonist CCL5 (25nmol/kg BW), as well as in combination with Sitagliptin Phosphate (50mg/kg BW) in lean male Swiss TO mice (n=6) were investigated. Before test compounds were administered, mice were fasted for 18 hrs and blood subsequently collected (t=0). Test compounds were administered orally and via intraperitoneal injection in glucose (18mmol/kg BW), and blood was collected at 15, 30, 60, 90 and 120 mins along with blood glucose measurements. Collected blood was centrifuged at 10000g for 5 mins at 4°C using a Beckman centrifuge (Beckman Instruments, UK). Plasma was stored at -20°C until insulin was measured by RIA as described in Chapter 2, section 2.2 and 2.3.

3.4: Results

3.4.1: Effects of CCL5 on insulin secretion from pancreatic BRIN-BD11 cells

The effects of CCL5 on insulin secretion from pancreatic BRIN-BD11 cells were tested in both 5.6mM and 16.7mM glucose (Figures 3.1 and 3.2). At 5.6mM glucose, CCL5 increased insulin secretion 1.3 to 1.8 fold (10^{-9} - 10^{-5} M, $p<0.05$ - $p<0.001$), Figure 3.1A). In hyperglycaemic conditions (Figure 3.2A), CCL5 increased insulin secretion 1.1 to 1.8-fold (10^{-8} - 10^{-5} M, $p<0.01$ - $p<0.001$). At both glucose conditions tested, CCL5 had no cytotoxic effects (Figures 3.1B and 3.2B).

3.4.2: Effects of CCL5 on insulin secretion from isolated islets

In mouse islets isolated from both lean and HFF Swiss TO mice, CCL5 increased insulin secretion from 10^{-10} to 10^{-6} M ($p<0.05$ - $p<0.001$). In lean islets, CCL5 increased secretion maximally at 10^{-6} M 3.3-fold compared to basal control ($p<0.001$, Figure 3.3A). This effect was demonstrated until

10^{-10}M ($p<0.05$) which augmented insulin secretion 1.3-fold. In HFF mouse islets, insulin secretion was increased 1.8-fold by CCL5 at 10^{-6}M ($p<0.001$, Figure 3.3B). Again this insulinotropic effect was maintained until 10^{-10}M , which increased insulin secretion 1.3-fold compared to glucose alone ($p<0.05$).

3.4.3: Distribution of GPR75 and insulin in BRIN-BD11 cells and lean and HFF NIH Swiss mouse pancreas

In order to determine the cellular localisation of GPR75 and insulin, double immunohistochemistry was carried out in BRIN-BD11 cells and lean and HFF mouse pancreas (Figure 3.4). GPR75 and insulin were found to be co-localised in pancreatic BRIN-BD11 cells (Figure 3.4D). The co-localisation of GPR75 and insulin was confirmed in lean and HFF mouse pancreas (Figure 3.4H and 3.4L). The effects of a high fat diet on NIH Swiss mice was calculated with a 36.9% increase ($p<0.01$) in GPR75 expression being observed in the pancreas of animals who received a high fat diet (Figure 3.5).

3.4.4: Distribution of GPR75 and glucagon in $\alpha\text{TC1.9}$ cells and lean and HFF NIH Swiss mouse pancreas

As with BRIN-BD11 cells, the co-localisation of GPR75 with glucagon was investigated in $\alpha\text{TC1.9}$ cells and lean and HFF NIH Swiss mouse pancreas. It was found that GPR75 and glucagon were not co-localised in lean or HFF mouse small intestine (Figures 3.6H and 3.6L), however it was found to be present in $\alpha\text{TC1.9}$ cells with glucagon (Figure 3.6D).

3.4.5: Distribution of GPR75 and GLP-1, GIP and PYY in intestinal cell lines and lean and HFF NIH Swiss mouse small intestine

The expression of GPR75 in the intestinal GLUTag and STC-1 cell lines as well as lean and HFF small intestine was examined using immunohistochemistry. Both GLP-1 and GPR75 were found throughout GLUTag cells (Figure 3.7D) and found to be co-localised in the intestinal cells of lean and HFF NIH Swiss mouse (Figures 3.7H and 3.7L).

GPR75 and GIP were found to be co-localised in the intestine, however not all GPR75 expressing cells were shown to be secreting GIP (Figures 3.9D and 3.9H). This was also seen for GPR75 and PYY which were found to be co-localised in both lean and HFF small intestinal tissue (Figures

3.10H and 3.10L). PYY and GPR75 were also found to be expressed throughout STC-1 cells (Figure 3.10D).

The effects of a high fat diet on GPR75 expression in the small intestine of NIH Swiss mice was determined using qPCR. Mice fed a high fat diet had a 46.37% reduction in GPR75 expression (Figure 3.8, $p < 0.05$).

3.4.6: Effects of CCL5 on GPR75 and insulin expression in BRIN-BD11 cells

In order to examine the effects of CCL5 treatment on GPR75 and insulin expression both qPCR and immunohistochemistry was carried out on BRIN-BD11 cells which had been treated for 20 mins and 4 hrs respectively. BRIN-BD11 cells treated with 5.6mM glucose demonstrated no change in insulin protein expression compared to media (Figures 3.11A and 3.11B). Treatment with CCL5 (10^{-6} M) in 5.6mM glucose resulted in a 69.1% increase in expression when compared to media ($p < 0.01$). Compared to 5.6mM glucose vehicle a 195.0% increase was observed ($p < 0.001$). Treatment with 16.7mM glucose resulted in a downregulation of insulin expression with a 60.1% reduction observed when compared to media ($p < 0.001$). BRIN cells treated with CCL5 (10^{-6} M) in 16.7mM glucose resulted in no change in insulin expression when compared to cells treated with media. When compared to 16.7mM glucose vehicle, CCL5 treatment resulted in a 292.0% increase in insulin protein expression ($p < 0.001$).

When BRIN-cells were treated for 4 hrs, all treatments downregulated insulin gene expression, with CCL5 treatment resulting in the largest downregulation at both 5.6 and 16.7mM glucose (Figures 3.12A and 3.12B). Treatment with 5.6 and 16.7mM glucose resulted in downregulation of 87.1% and 91.3% when compared to untreated cells respectively ($p < 0.001$). Treatment with CCL5 in both concentrations resulted in downregulation of 94.0% in 5.6mM glucose while a downregulation of 95.8% was observed in 16.7mM glucose compared to media ($p < 0.001$). When compared to the vehicle controls, there was a 53.7% reduction in insulin protein expression from cells treated with CCL5 in 5.6mM glucose ($p < 0.01$), while a 51.5% reduction was observed in cells treated with CCL5 in 16.7mM glucose ($p < 0.05$).

Treatment of BRIN-BD11 cells for 20 mins resulted in downregulated GPR75 expression in all but one condition when compared to untreated cells (Figures 3.14A and 3.14B). Cells treated with 10^{-6} M CCL5 in 5.6mM glucose were upregulated 43.1% ($p < 0.001$). When compared to the vehicle control, this upregulation was increased to 222.0% ($p < 0.001$) as treatment of BRIN-BD11 cells with 5.6mM glucose resulted in a 55.6% reduction when compared to cells which were treated with media ($p < 0.001$). Treatment with 16.7mM glucose reduced GPR75 expression 71.9% ($p < 0.001$), however the presence of CCL5 in this glucose concentration increased GPR75 expression 86.0%

($p < 0.001$). When GPR75 expression levels of BRIN-BD11 cell treatment with CCL5 in hyperglycaemic conditions was compared to untreated cells, a 47.0% reduction was observed ($p < 0.001$).

BRIN-BD11 cells treated for 4 hrs resulted in reduced GPR75 expression in all conditions tested (Figures 3.15A and 3.15B). Both 5.6mM and 16.7mM glucose had lower expression levels than those demonstrated when cells were also supplemented with CCL5. In normoglycaemic conditions, a 85.6% downregulation was observed while a reduction of 85.8% was seen in 16.7mM glucose when compared to untreated cells ($p < 0.001$). BRIN-BD11 levels treated with CCL5 in 5.6mM glucose demonstrated a downregulation of 77.1% compared to the expression level seen in cells treated with media ($p < 0.001$). This was a 1.6-fold increase in GPR75 expression when compared to the vehicle control ($p < 0.05$). Meanwhile treatment of cells with CCL5 in 16.7mM glucose resulted in a 78.7% reduction in GPR75 expression ($p < 0.001$) which was a 1.5-fold increase when compared to the 16.7mM glucose treatment alone ($p < 0.01$).

qPCR on cells treated for 4 hrs demonstrated reduced insulin expression (Figure 3.13). Treatment of BRIN-BD11 cells resulted in greater insulin gene expression downregulation when compared with the vehicle control. Both 5.6mM and 16.7mM glucose reduced insulin gene expression 46.0% ($p < 0.05$) and 40.0% ($p < 0.05$), compared to untreated cells. Treatment with CCL5 in 5.6mM glucose resulted in downregulation of 41.3% ($p < 0.05$) when compared to vehicle control. Treatment with CCL5 at 16.7mM glucose had no effect.

3.4.7: Effects of CCL5 on proglucagon and PC1 gene expression in intestinal GLUTag cells

In order to assess the effects of CCL5 treatment on GLP-1 expression, GLUTag cells were treated with CCL5 (10^{-6} M, Figure 3.16A and 3.16B). Cells treated with 2mM glucose were upregulated 14-fold while those treated with CCL5 were upregulated 41-fold when compared to untreated cells ($p < 0.001$) (Figure 3.16A). When compared to cells treated with glucose, CCL5 increased proglucagon gene expression 2-fold ($p < 0.05$). The proglucagon gene is processed by PC1/3 in the small intestine and treatment of GLUTag cells with CCL5 resulted in a 1.8-fold increase compared to untreated cells (Figure 3.16B, $p < 0.05$). Compared to the 2mM glucose vehicle control PC1 expression is upregulated 2.3-fold ($p < 0.05$) (Figure 3.16B).

3.4.8: Acute effects of GPR39 agonists on blood glucose and insulin in fasted male Swiss TO mice

In order to test the effects of GPR75 activation *in vivo*, CCL5 was administered both orally and via IP injection to male fasted Swiss TO mice. CCL5 was also administered orally with the DPP-IV

inhibitor Sitagliptin Phosphate. IP administration of 25nmol/kg BW CCL5 resulted in reduced blood glucose from 30 to 60 mins post administration (Figure 3.17A, $p<0.001$). A 1.5-fold reduction in blood glucose was observed after 30 mins and this was sustained after 60 mins. The AUC (Figure 3.17B) confirmed an overall reduction of 1.3-fold ($p<0.001$). Insulin secretion was enhanced by 41.6% (Figure 3.18A, $p<0.001$) 15 mins post administration. This effect was sustained 30 mins post injection with an increase of 41.7% being observed. Overall, CCL5 increased insulin secretion in lean mice by 40.1% as measured by AUC (Figure 3.18B, $p<0.001$).

Oral administration of 25nmol/kg BW CCL5 (Figure 3.19A) resulted in a reduction in blood glucose from 15-120 mins ($p<0.01$ - $p<0.001$). The greatest reduction in blood glucose was seen 90 mins post administration with a 49.8% reduction being observed (Figure 3.19A). Blood glucose was reduced by 39.8%, 47.1%, 39.3% and 47.4% at 15, 30, 60 and 120 mins, respectively post administration. Like CCL5 administration alone, Sitagliptin in combination with CCL5 reduced blood glucose over the course of the testing period with reductions of 39.8%, 43.2%, 30.6%, 41.6% and 31.0% (Figure 3.19A, $p<0.05$ - $p<0.001$). However compared to CCL5 treatment alone there was an overall increase in blood glucose of 7.5% (Figure 3.19B). Overall there was a 41.9% reduction in blood glucose as determined by the AUC ($p<0.001$). Administration of Sitagliptin alone reduced blood glucose over the course of the study. Blood glucose was reduced 20.0%, 34.1%, 27.1%, 37.1% and 34.4% at 15, 30, 60, 90 and 120 mins respectively (Figure 3.19A). Compared to Sitagliptin and CCL5 there was a 24.8% increase in blood glucose 15 mins post administration ($p<0.05$) and this effect was seen overall in the AUC ($p<0.001$).

The largest increase in insulin secretion was observed in mice treated with CCL5 alone (Figure 3.20A). Insulin secretion was increased 2.2-fold ($p<0.001$) 30 minutes post administration when compared to glucose administration alone, while this effect was sustained until 60 mins with a 1.6-fold increase being observed ($p<0.05$). CCL5 administered alongside Sitagliptin also increased insulin secretion (1.9-fold increase) 30 mins post administration ($p<0.001$). Sitagliptin administration alone resulted in a 1.3-fold increase in insulin secretion after 15 mins, compared to glucose administration. Both CCL5 and CCL5 in combination with Sitagliptin outperformed Sitagliptin treatment alone with 1.8 and 1.5-fold increases in secretion being observed after 30 mins ($p<0.001$).

3.5: Discussion

GPR75 was first identified in the human genome in the eye (Tarttelin *et al.* 1999) and so to date much research has explored the effects of GPR75 activation on eye disorders (Tarttelin *et al.* 1999, Sauer *et al.* 2001). The first identified GPR75 agonist was CCL5 (Ignatov *et al.* 2006), a proinflammatory cytokine (Keane, Strieter 2000) that had previously been identified as playing a

role in experimental autoimmune uveitis (Crane *et al.* 2001) as well as the recruitment of immune cells to β -cells of the pancreas in Type 1 Diabetes (Carvalho-Pinto *et al.* 2004). Interestingly, CCL5 is degraded by DPP-IV (Oravecz *et al.* 1997) and the degraded form of CCL5 reduces the ability of HIV to infect the body (Schols *et al.* 1998). A second GPR75 agonist has been identified recently (Garcia *et al.* 2017).

Previous research has shown that GPR75 activation can play a role in glucose homeostasis (Liu *et al.* 2013). This study investigated the *in vitro* and *in vivo* effects of GPR75 activation by CCL5 on insulin secretion as well as the effects on gastrointestinal hormone expression. A dose dependent increase in insulin secretion was observed in BRIN-BD11 cells and isolated islets from lean and HFF mice treated with CCL5. This confirms the insulin secretory effects described previously (Liu *et al.* 2013). The insulinotropic effect of CCL5 was greater in lean islets than those from HFF mice. Previous studies have shown that HFF mice have reduced glucose induced insulin secretion and while the insulin level is reduced when compared to total pancreas size, there was no significant difference between the insulin content of lean and HFF mice (Capito *et al.* 1992). Interestingly there were no cytotoxic effects observed in BRIN-BD11 cells as CCL5 has been shown to play a role in the destruction of β -cells in Type 1 Diabetes (Carvalho-Pinto *et al.* 2004) however it appears that this only occurs when an immune response to the body's β -cells is triggered.

Both GPR75 and CCL5 have been found to be co-localised with insulin and glucagon in the pancreas (Liu *et al.* 2013). In this study we confirmed the co-localisation of insulin and GPR75 in both lean and HFF pancreas as well as BRIN-BD11 cells showing that CCL5 activation of GPR75 is an autocrine effect. However, we were unable to find any co-localisation of GPR75 and glucagon in the α -cells of the intestine. Feeding mice a high fat diet led to increased GPR75 gene expression making it a promising target in the treatment of Type 2 Diabetes which is closely linked with obesity (Kahn, Hull & Utzschneider 2006). Treatment of BRIN-BD11 cells with CCL5 resulted in upregulation of insulin and GPR75 protein expression. However, qPCR studies found that insulin expression was downregulated while GPR75 expression was still upregulated at both 5.6mM and 16.7mM glucose. There may be a number of factors involved, however it appears that the increased insulin secretory effect of CCL5 treatment causes the initial upregulation of insulin but cells are not able to maintain the production of insulin and existing stocks become exhausted. The initial downregulation of insulin by both 5.6 and 16.7mM glucose provides further evidence of this as initially insulin production is reduced. The protein expression studies using immunocytochemistry were confirmed using qPCR. Meanwhile GPR75 production will still be increased after 4 hours as the receptor protein will not be secreted. Rather the receptor will become internalised, repackaged and transported back to the membrane.

There is currently limited information on the presence of GPR75 in the intestine. Intestinal endocrine cells make up 1% of the small intestine (Mace, Tehan & Marshall 2015) and there is currently no published research showing the presence of GPR75. Using immunohistochemistry and qPCR, the presence of GPR75 in the K and L cells of the intestine were identified. qPCR demonstrated the presence of GPR75 in the small intestine, with downregulation when mice were fed a high fat diet. Both GLP-1 and PYY were co-localised with GPR75 in the L-cells of the intestine while GIP was co-localised with GPR75 in the K-cells of the intestine. Staining in both GLUTag and STC-1 cells confirmed the co-localisation seen *ex vivo*. Treatment of GLUTag cells with CCL5 also resulted in upregulation of the proglucagon gene along with the PC1 gene which processes the proglucagon gene into GLP-1 in the small intestine. This suggests that CCL5 treatment will increase GLP-1 secretion from L-cells of the intestine however this would need explored.

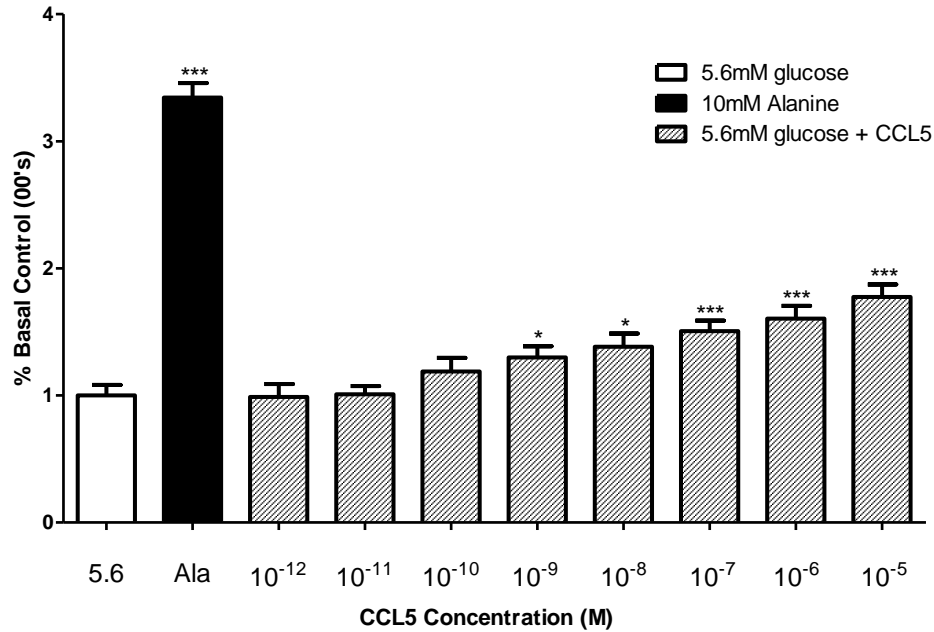
To further confirm the *in vitro* findings, fasted male Swiss TO mice were administered 25nmol/kg body weight CCL5 both orally and via IP injection. This concentration of CCL5 was selected as it is the concentration used in our lab previously for peptides used in vivo studies (Moffett *et al.* 2015). As DPP-IV, an enzyme that degrades GLP-1 and GIP (Hansen *et al.* 1999, Deacon 2004), also degrades CCL5 (Oravecz *et al.* 1997), oral administration of the agonist was also carried out in combination with the DPP-IV inhibitor Sitagliptin Phosphate. Sitagliptin has previously been used in combination studies in such as with Metformin (Shannon *et al.* 2009), pioglitazone (Alba *et al.* 2009 and Bailey *et al.* 2010) and glimepiride (Ishii *et al.* 2014). Previously CCL5 has shown to have glucose lowering and insulinotropic effects *in vivo* (Liu *et al.* 2013). This study confirms the previous effects seen *in vivo* with IP administration reducing blood glucose and increasing insulin secretion from the pancreas. Interestingly CCL5 had glucose reducing and insulinotropic actions when administered orally both alone and in combination with Sitagliptin. The evidence presented in this study suggests that CCL5 is present in the enteroendocrine cells of the intestine and this opens this area for further investigation. Sitagliptin administration with CCL5 also performed better than Sitagliptin treatment alone. There is precedent for the cleaved form of CCL5 performing better than the intact form as cleaved CCL5 has been shown to be better at protecting from HIV protection (Proost *et al.* 1998). Further work using the cleaved form of CCL5 needs to be carried out to elucidate whether this is the case including *in vitro* studies. As CCL5 recruits T cells to places of inflammation including to the pancreas (Carvalho-Pinto *et al.* 2004) the long term effects of CCL5 treatment on peripheral tissues will need to be investigated further. Furthermore the conflicting evidence on CCL5 binding to GPR75 mean further studies including GPR75 knockdown and GPR75 knockout will need to be carried out to further elucidate the effects of CCL5 on this receptor.

In conclusion, this study has determined that CCL5 increases insulin secretion *in vitro* and *in vivo*. This confirmed previous research in this area (Liu *et al.* 2013). GPR75 was also found to be present

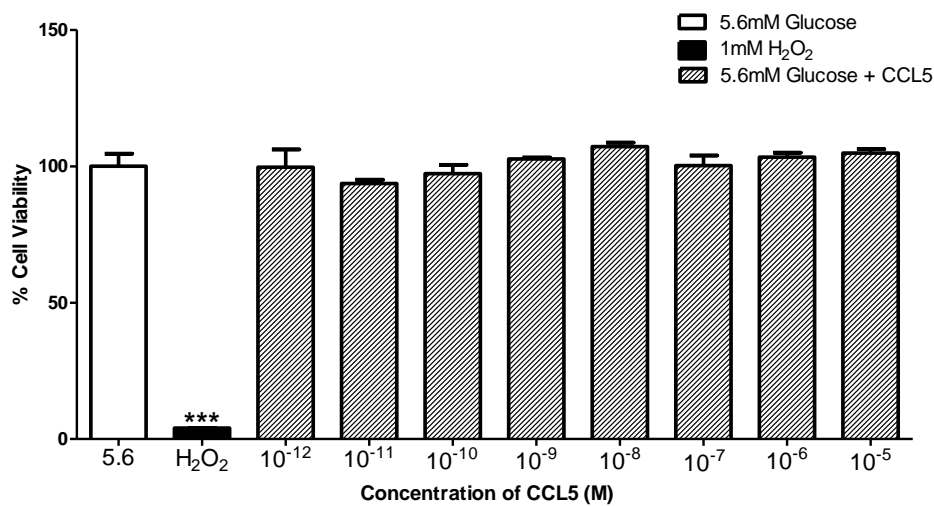
in the endocrine cells of the intestine, namely the L and K –cells. Treatment of GLUTag cells with CCL5 leads to increased proglucagon and PC1 expression suggesting GPR75 activation has a role in the regulation of incretin hormone secretion. Oral administration of CCL5 reduced blood glucose however when used in combination with Sitagliptin this effect was reduced, suggesting that DPP-IV cleavage of CCL5 may increase its effectiveness as an antidiabetic therapy. These results indicate that GPR75 may play a role in incretin and insulin secretion and may be a possible therapy in the treatment of Type 2 Diabetes.

Figure 3.1: Effects of GPR75 agonist CCL5 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



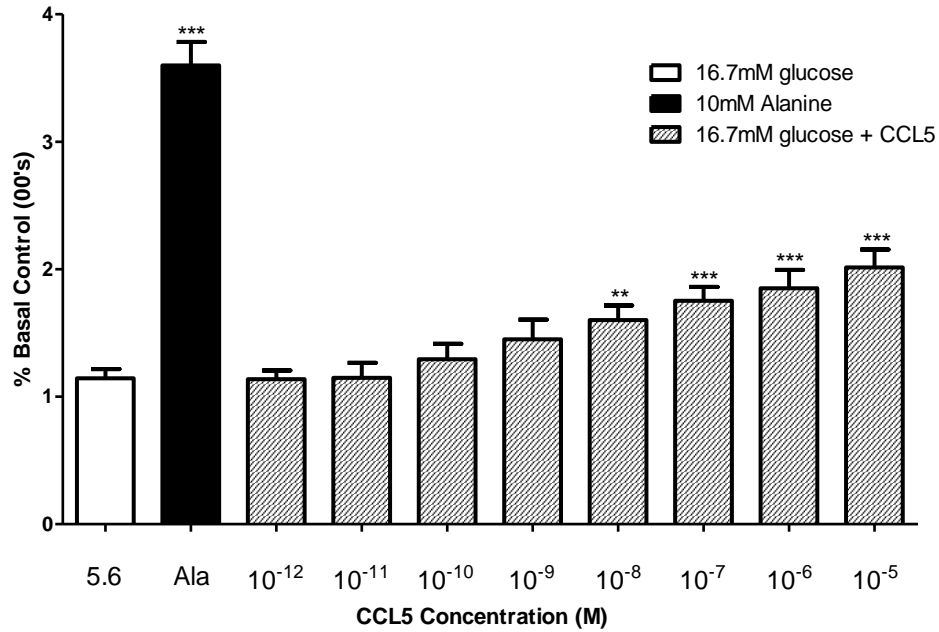
B.



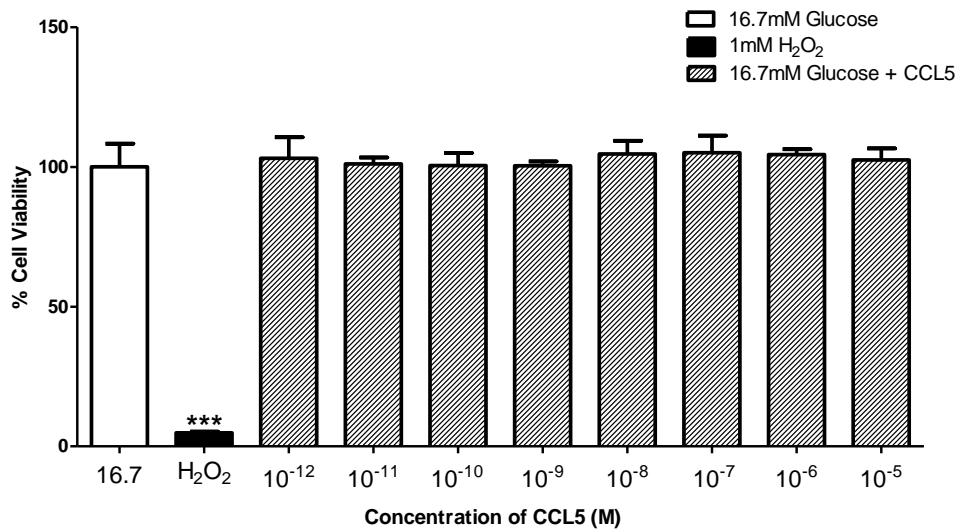
Effect of CCL5 (10^{-12} to 10^{-5} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using MTT Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$ and *** $p < 0.001$, compared to basal glucose control for insulin secretion and 1mM H₂O₂ toxic control for cell viability.

Figure 3.2: Effects of GPR75 agonist CCL5 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.



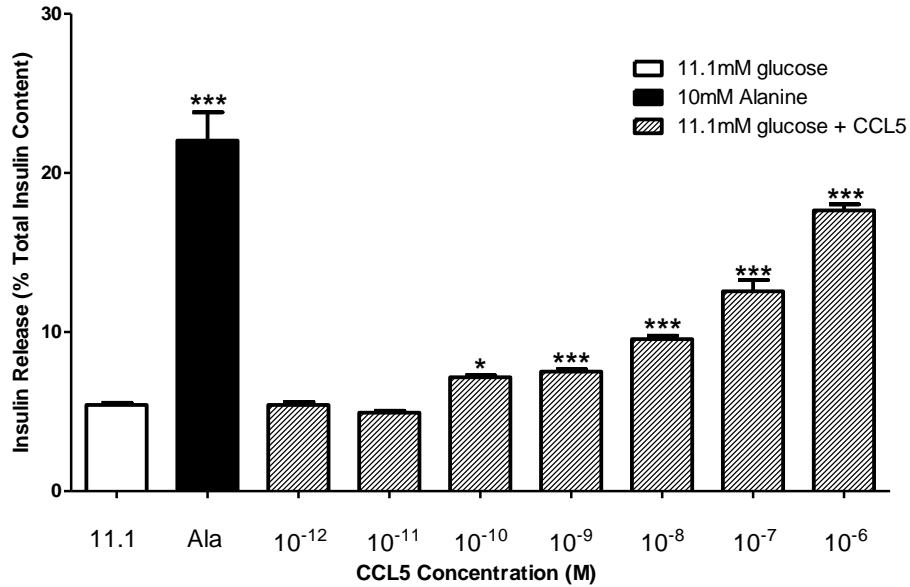
B.



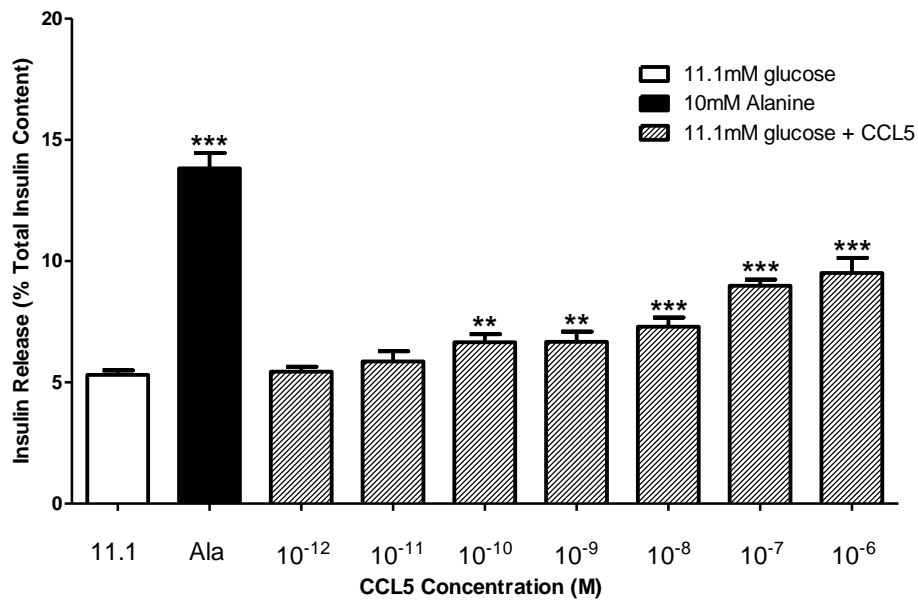
Effect of CCL5 (10^{-12} to 10^{-5} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using MTT Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. **p<0.01 and ***p<0.001 compared to basal glucose control for insulin secretion and 1mM H₂O₂ toxic control for cell viability.

Figure 3.3: Effect of GPR75 agonist CCL5 on insulin secretion from isolated islets from lean and HFF Swiss TO mice

A.

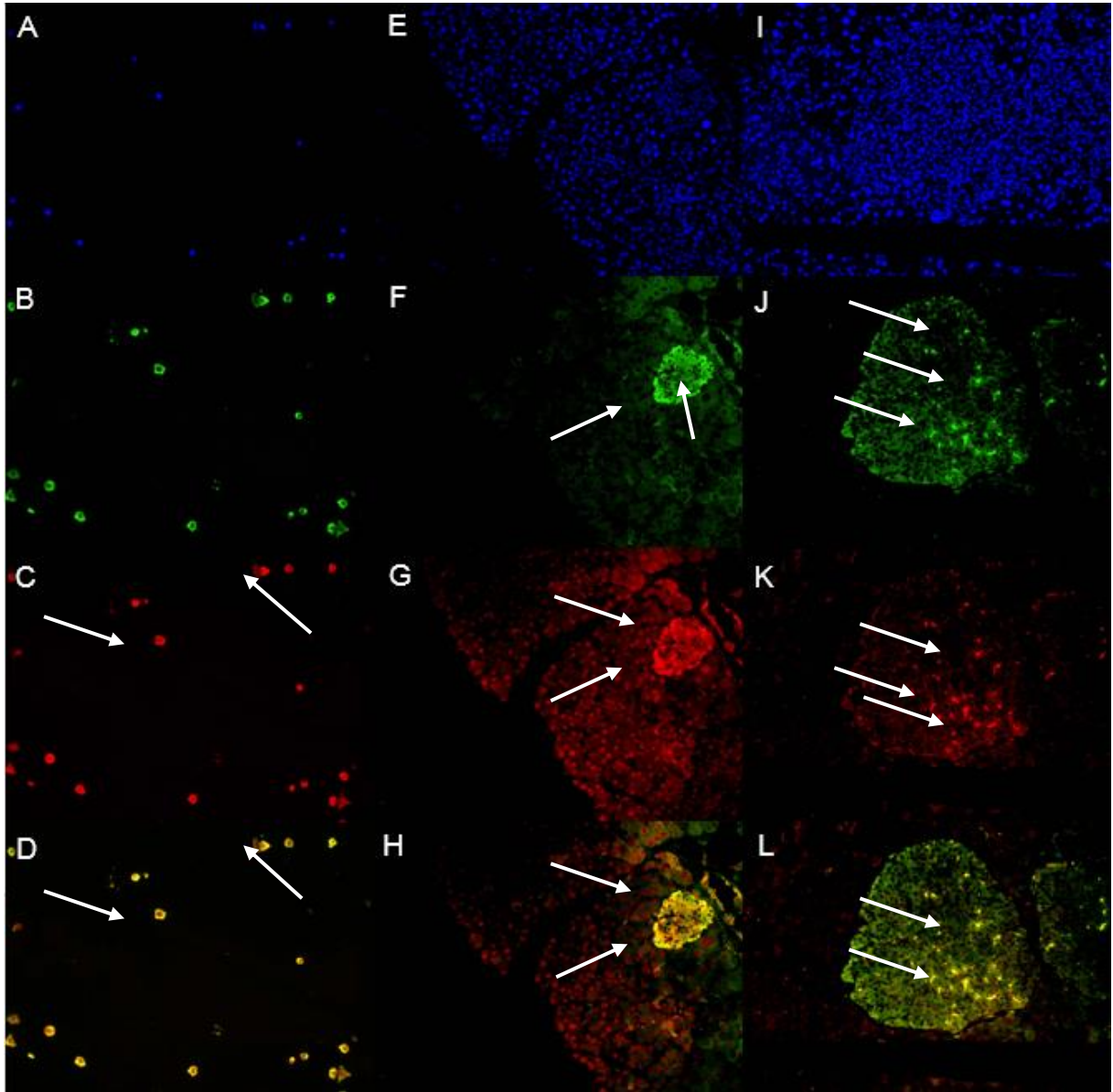


B.



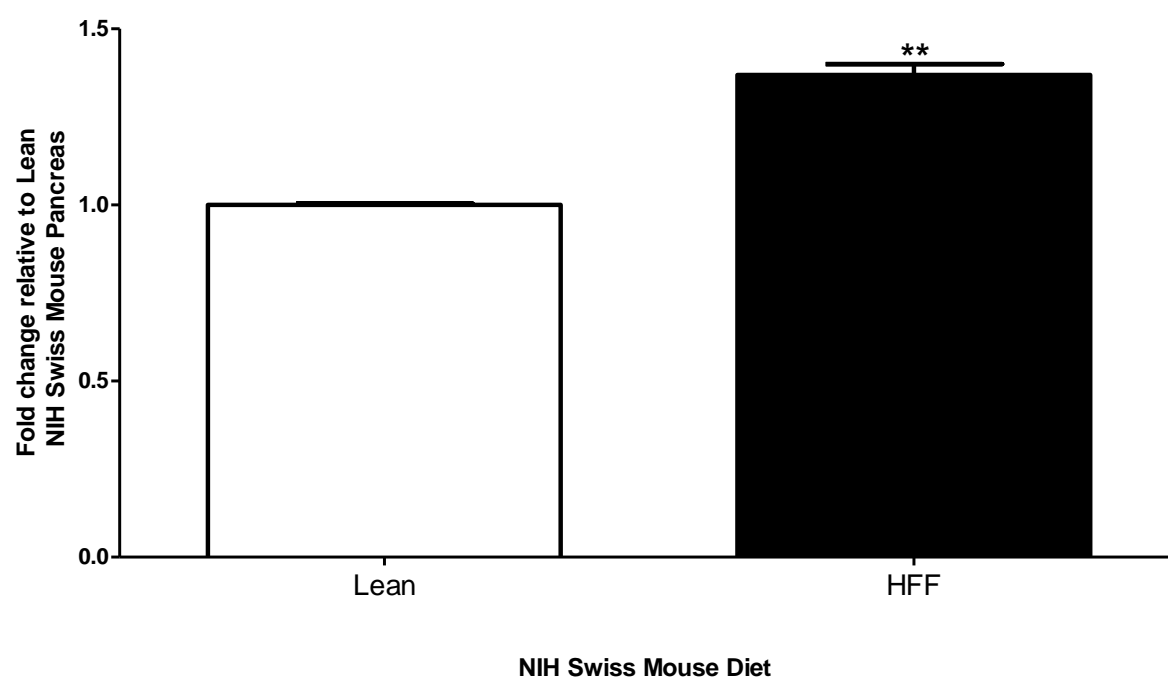
Effect of CCL5 on insulin secretion from isolated islets from lean (A) and HFF (B) Swiss TO mice in 11.1mM glucose. Results are the mean \pm SEM (n=4). *p<0.05, **p<0.01 and ***p<0.001, compared to the basal control.

Figure 3.4: Double immunofluorescence staining of GPR75 and insulin in BRIN-BD11 cells and lean and HFF NIH Swiss mouse pancreas



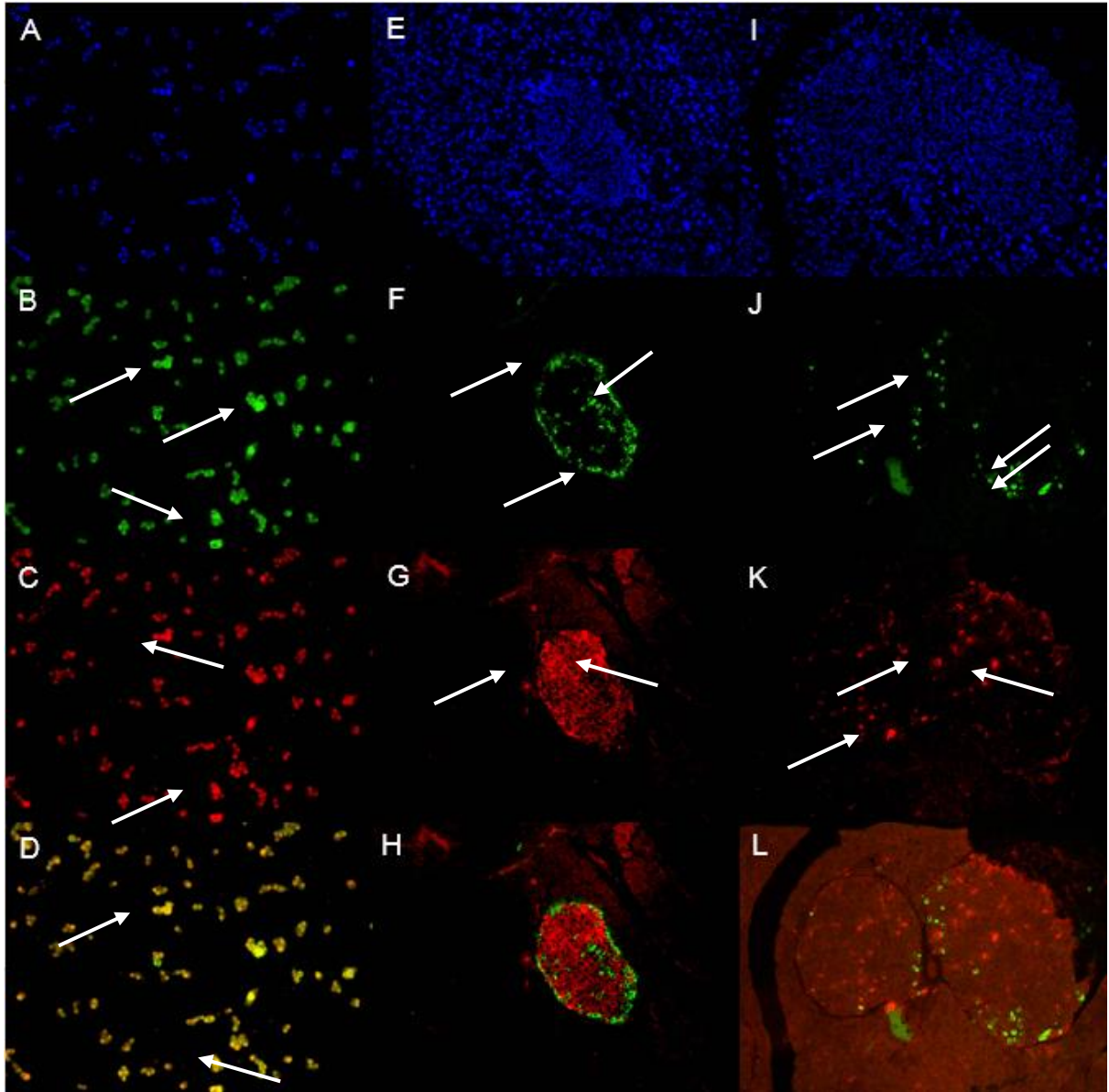
Distribution of DAPI (A, E, I), insulin (B, F, J), GPR75 (C, G, K) and double immunofluorescence (D, H, L) of insulin and GPR75 in BRIN-BD11 cells (A-D), lean (E-H) and HFF (I-L) NIH Swiss mouse tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 3.5: GPR75 expression in lean and HFF male NIH Swiss mouse pancreas



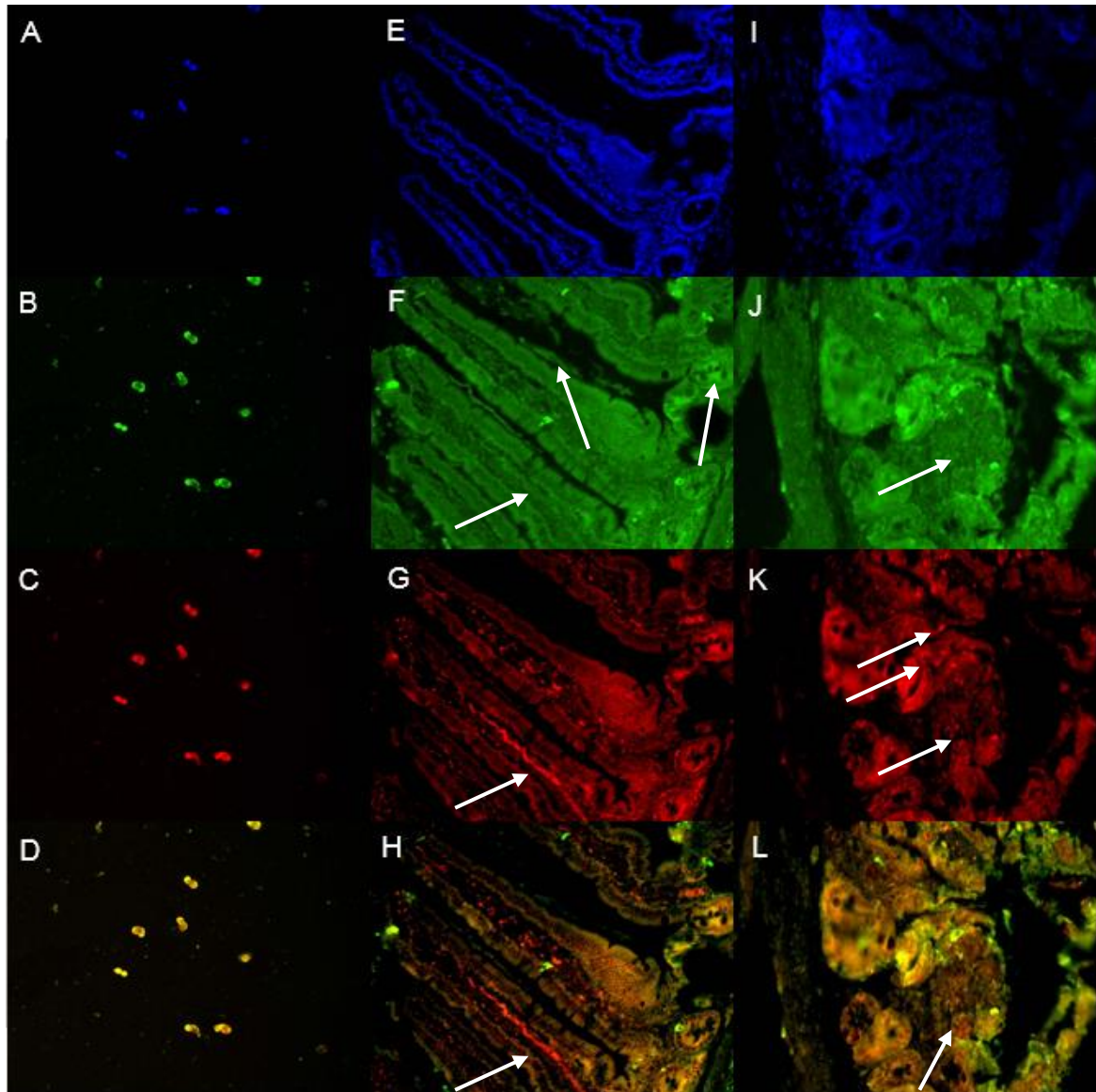
Effect of HFF diet on GPR75 gene expression in male NIH Swiss Mouse Pancreas (n=6). ** p<0.01, compared to lean pancreas.

Figure 3.6: Double immunofluorescence staining of GPR75 and glucagon in α TC1.9 cells and lean and HFF NIH Swiss mouse pancreas



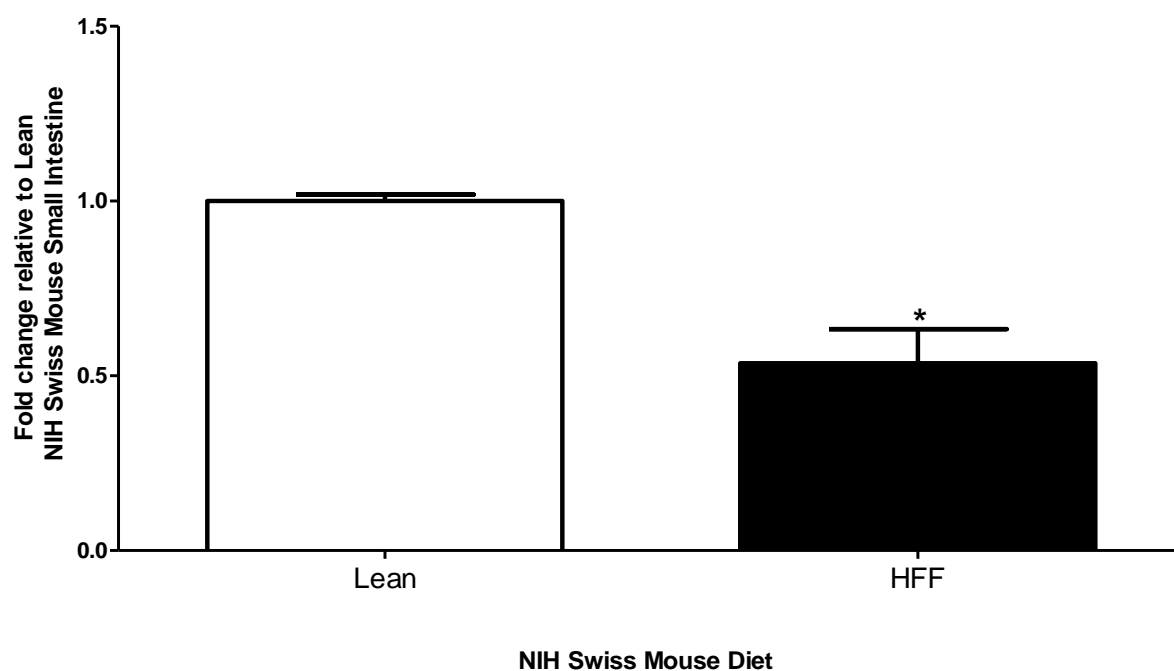
Distribution of DAPI (A, E, I), glucagon (B, F, J), GPR75 (C, G, K) and double immunofluorescence (D, H, L) of glucagon and GPR75 in α TC1.9 cells (A-D), lean (E-H) and HFF (I-L) NIH Swiss mouse tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 3.7: Double immunofluorescence staining of GLP-1 and GPR75 in GLUTag cells and lean and HFF NIH Swiss mouse small Intestine



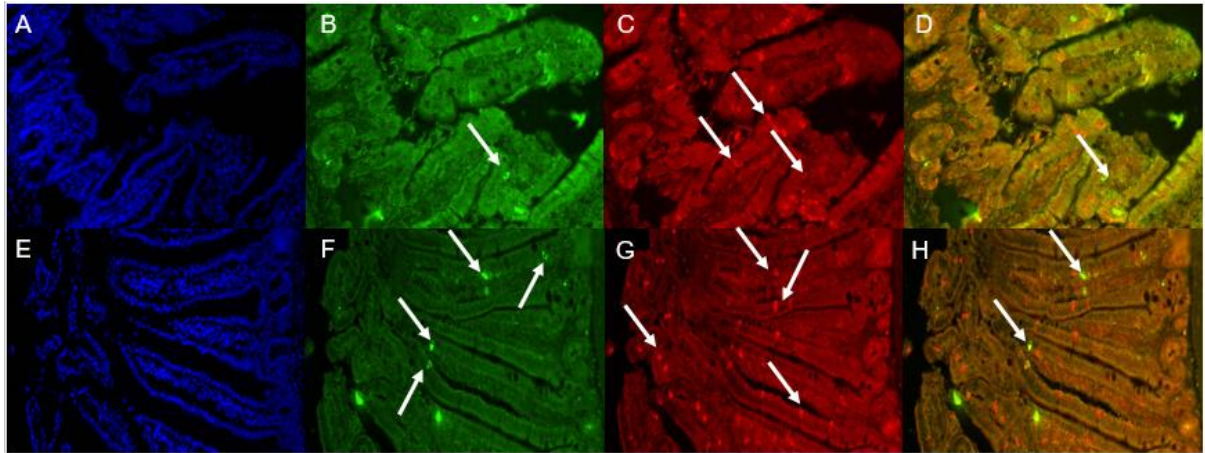
Distribution of DAPI (A, E, I), GLP-1 (B, F, J), GPR75 (C, G, K) and double immunofluorescence (D, H, L) of GLP-1 and GPR75 in GLUTag cells (A-D), lean (E-H) and HFF (I-L) NIH Swiss mouse tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 3.8: GPR75 gene expression in lean and HFF male NIH Swiss mouse small intestine



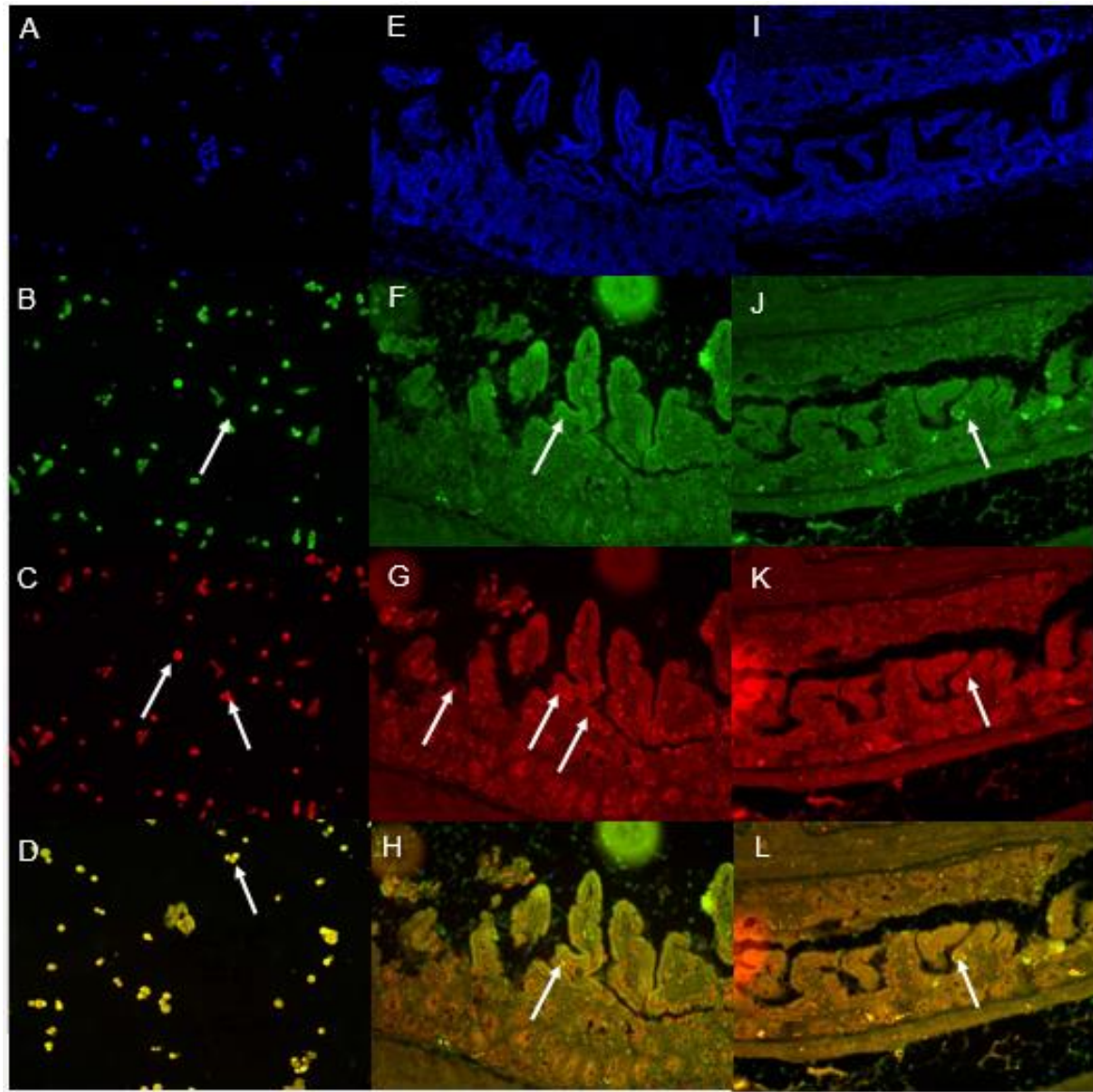
Effect of HFF diet on GPR75 gene expression in male NIH Swiss mouse small intestine (n=6). *
p<0.05, compared to lean mice.

Figure 3.9: Double immunofluorescence staining of GIP and GPR75 in pGIPneo STC-1 cells and lean and HFF NIH Swiss mouse small intestine



Distribution of DAPI (A and E), GIP (B and F), GPR75 (C and G) and double immunofluorescence (D and H) of GIP and GPR75 in lean (A-D) and HFF (E-H) NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

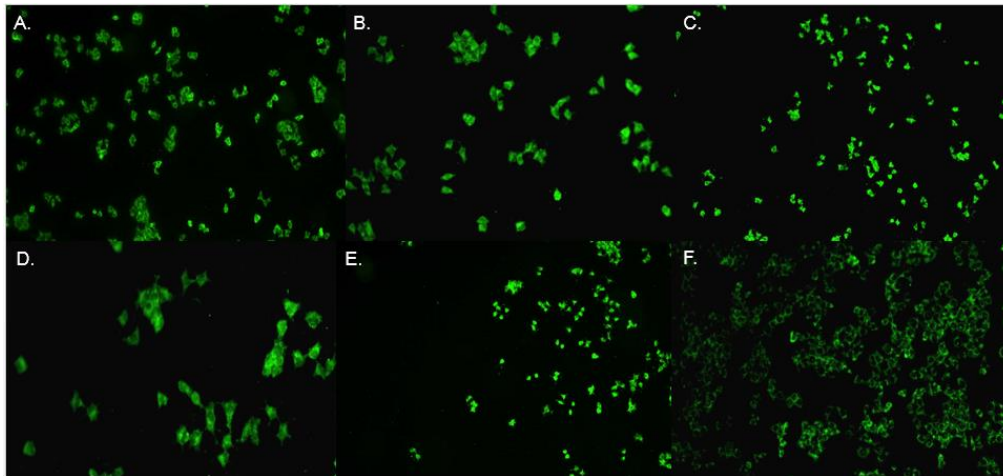
Figure 3.10: Double immunofluorescence staining of PYY and GPR75 in STC-1 cells and lean and HFF NIH Swiss mouse small intestine



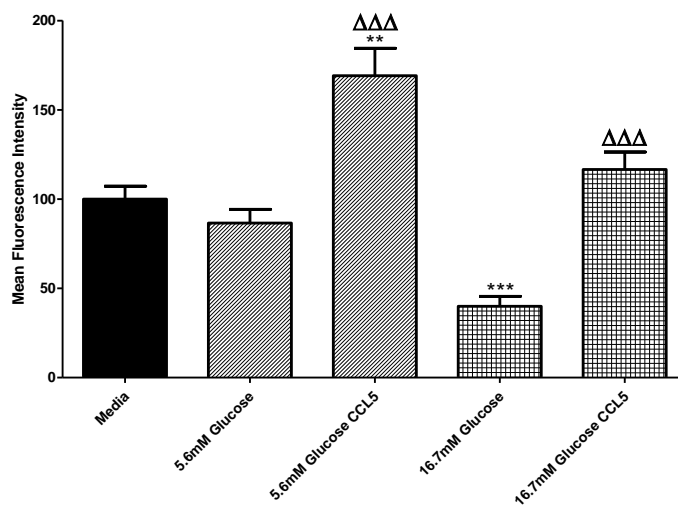
Distribution of DAPI (A, E, I), PYY (B, F, J), GPR75 (C, G, K) and double immunofluorescence (D, H, L) of PYY and GPR75 in STC-1 cells (A-D), lean (E-H) and HFF (I-L) NIH Swiss mouse tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 3.11: Effect of 20 min CCL5 treatment on insulin expression in BRIN-BD11 Cells

A.



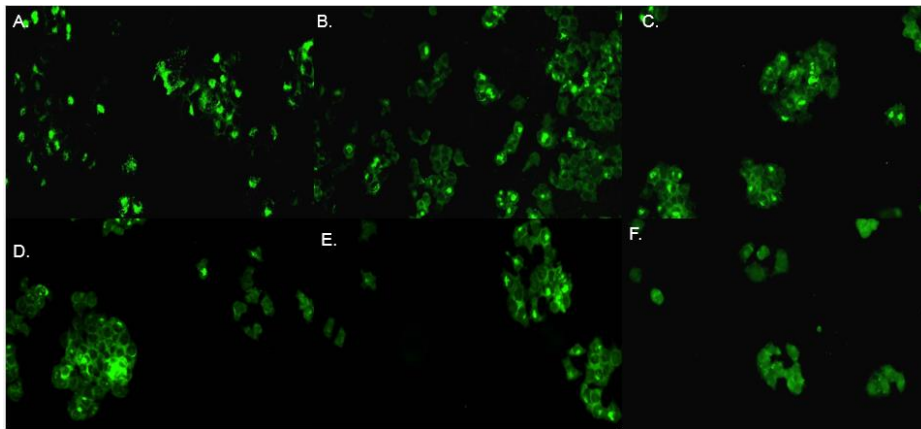
B.



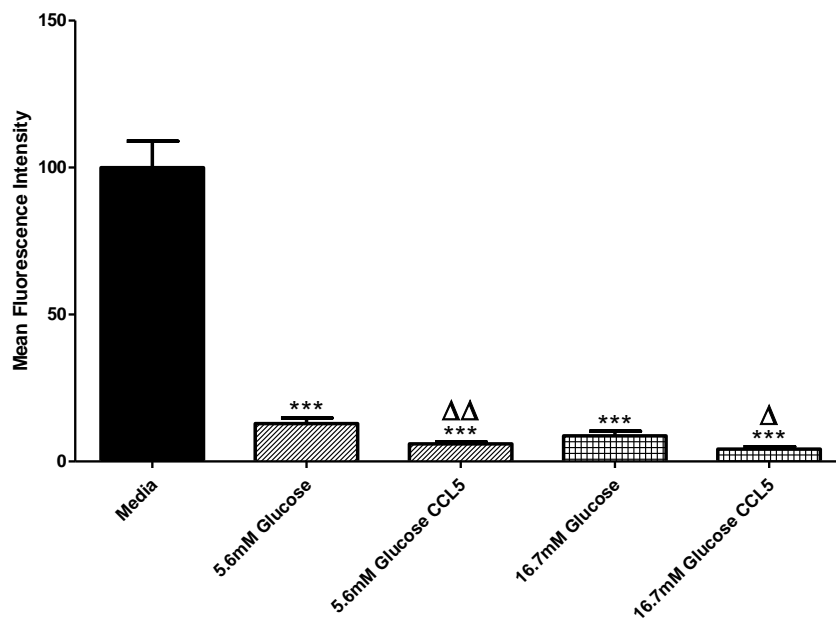
Effects of CCL5 on insulin protein expression after 20 min treatment in pancreatic BRIN-BD11 cells in 5.6mM and 16.7mM glucose (n=3). A – media; B – 5.6mM glucose; C – 5.6mM glucose + CCL5; D – 16.7mM glucose; E. 16.7mM glucose + CCL5; F – negative control without primary antibody. Intensity values for negative control without primary antibody were subtracted from the fluorescence intensity values obtained for test conditions. ** $p < 0.01$ and *** $p < 0.001$ compared to untreated cells. ΔΔΔ $p < 0.001$ compared to vehicle control.

Figure 3.12: Effect of 4 hr CCL5 treatment on insulin expression in BRIN-BD11 Cells

A.

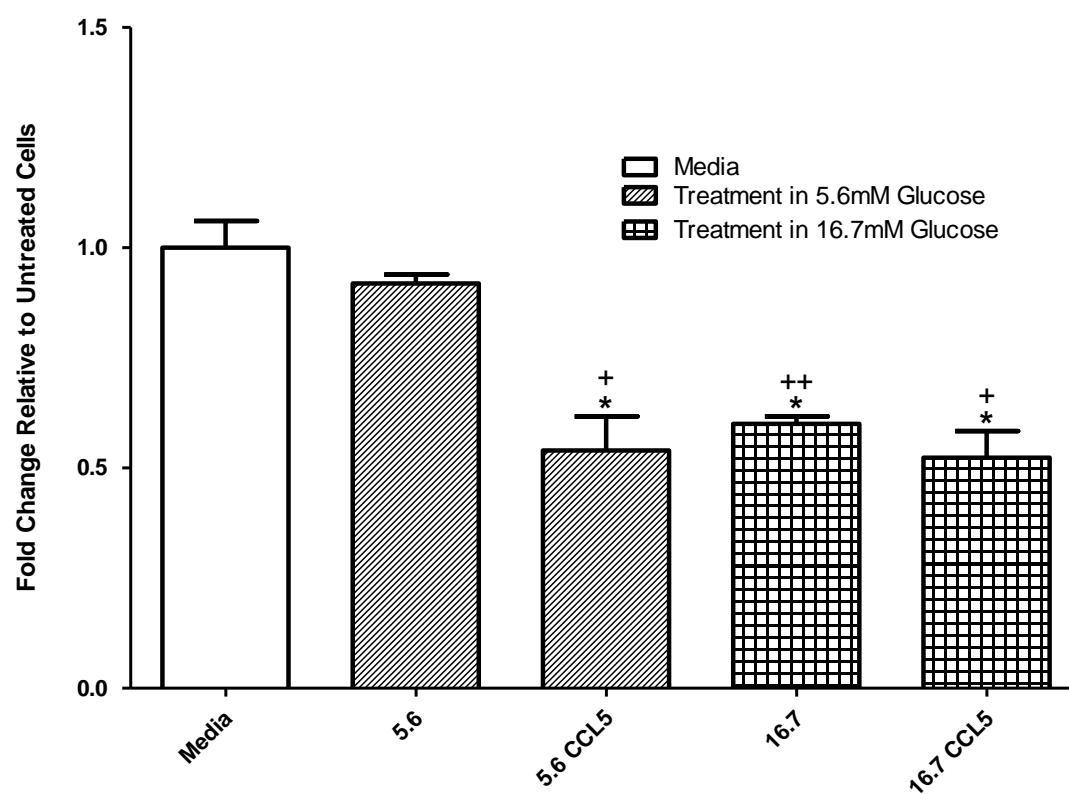


B.



Effects of CCL5 on Insulin gene expression after 4 hr treatment in pancreatic BRIN-BD11 cells in 5.6mM and 16.7mM glucose (n=3). A – media; B – 5.6mM glucose; C – 5.6mM glucose + CCL5; D – 16.7mM glucose; E. 16.7mM glucose + CCL5; F – negative control without primary antibody. Intensity values for negative control without primary antibody were subtracted from the fluorescence intensity values obtained for test conditions. *** p<0.001 compared to untreated cells. Δ p<0.05 and ΔΔ p<0.01 compared to vehicle control.

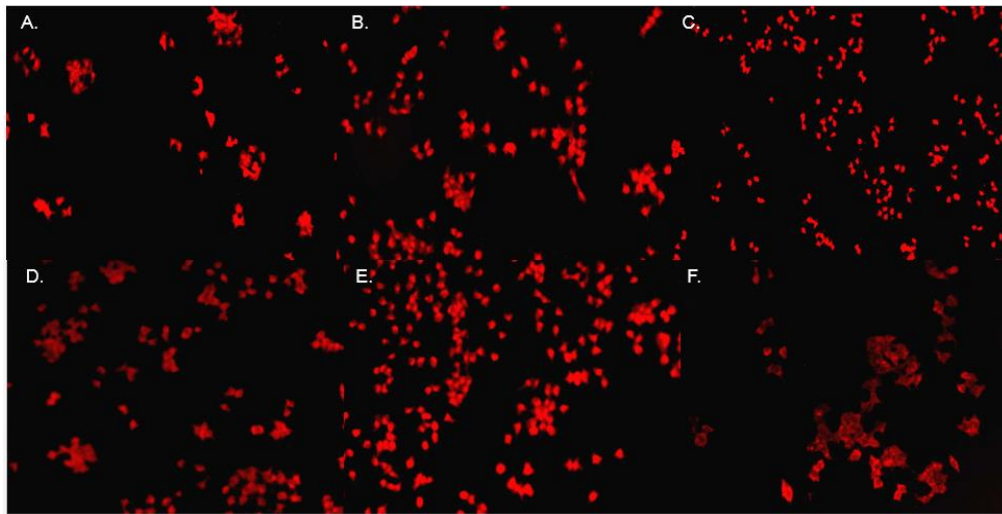
Figure 3.13: Effect of 4 hr CCL5 treatment on insulin expression in BRIN-BD11 Cells measured by qPCR



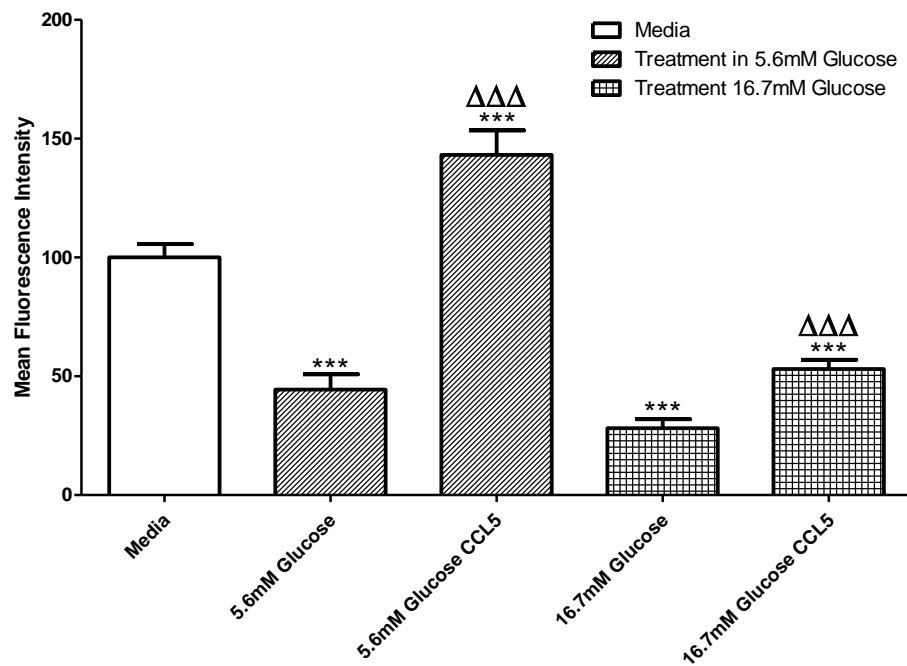
Effects of CCL5 on Insulin gene expression after 4 hour treatment in pancreatic BRIN-BD11 cells in 5.6mM and 16.7mM glucose (n=3). * $p<0.05$ compared to untreated cells. + $p<0.05$ and ++ $p<0.01$ compared to 5.6mM glucose.

Figure 3.14: Effect of 20 min CCL5 treatment on GPR75 expression in BRIN-BD11 Cells

A.



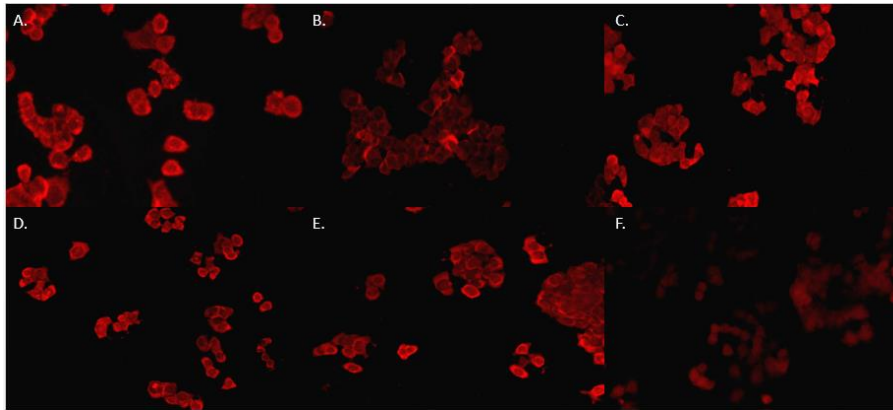
B.



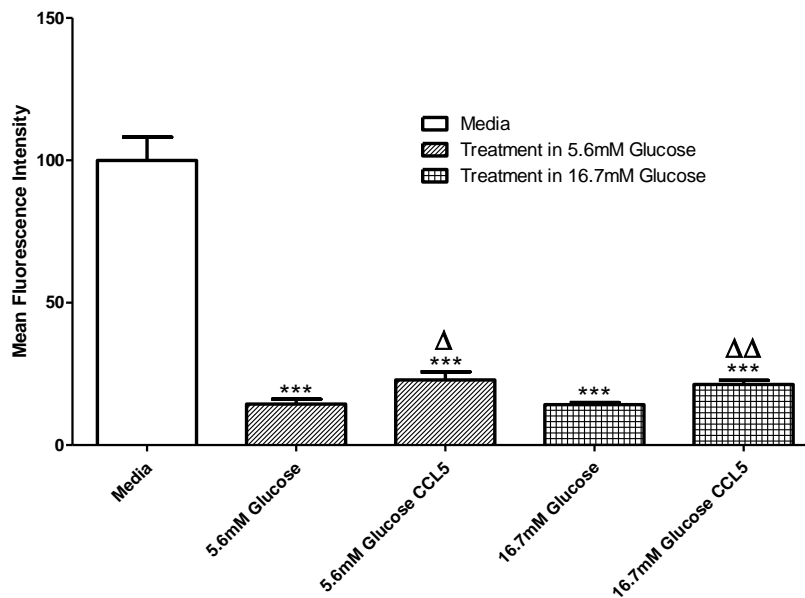
Effects of CCL5 on GPR75 protein expression after 20 min treatment in pancreatic BRIN-BD11 cells in 5.6mM and 16.7mM glucose (n=3).. A – media; B – 5.6mM glucose; C – 5.6mM glucose + CCL5; D – 16.7mM glucose; E. 16.7mM glucose + CCL5; F – negative control with no primary antibody. Intensity values for negative control without primary antibody were subtracted from the fluorescence intensity values obtained for test conditions. *** p<0.001 compared to untreated cells. ΔΔΔ p<0.001 compared to vehicle control.

Figure 3.15: Effect of 4 hr CCL5 treatment on GPR75 expression in BRIN-BD11 Cells

A.



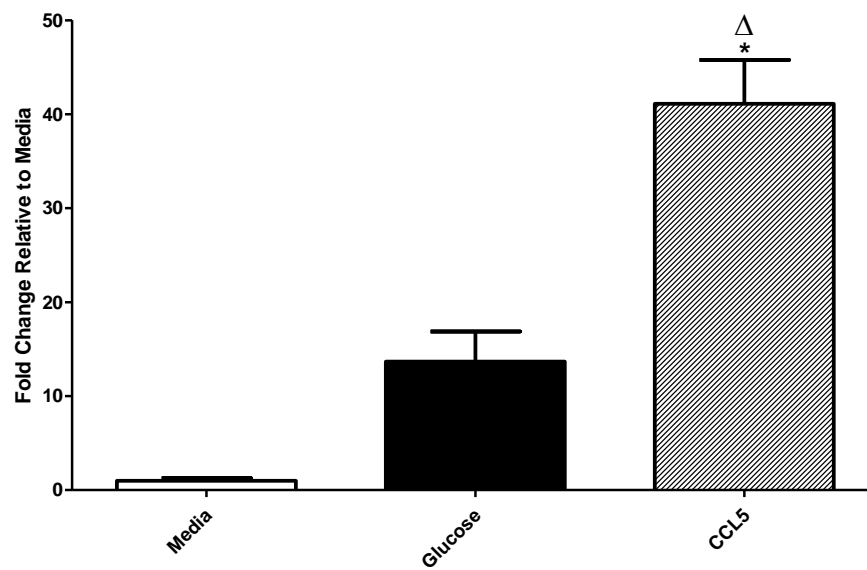
B.



Effects of CCL5 on GPR75 protein expression after 4 hour treatment in pancreatic BRIN-BD11 cells in 5.6mM and 16.7mM glucose (n=3). A – media; B – 5.6mM glucose; C – 5.6mM glucose + CCL5; D – 16.7mM glucose; E. 16.7mM glucose + CCL5; F – negative control with no primary antibody. Intensity values for negative control without primary antibody were subtracted from the fluorescence intensity values obtained for test conditions. *** p<0.001 compared to untreated cells. Δ p<0.05 and ΔΔ p<0.01 compared to vehicle control.

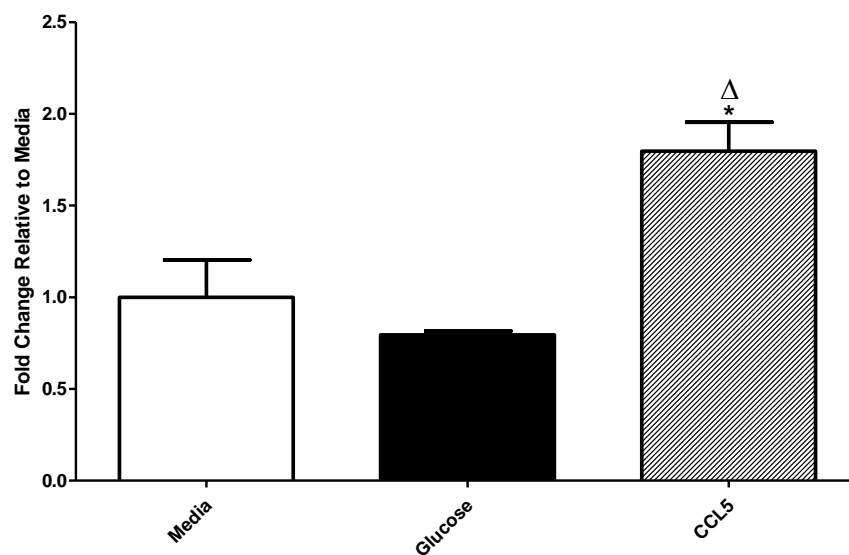
Figure 3.16: Effect of CCL5 on proglucagon and PC1 gene expression in GLUTag cells

A.



Effects of CCL5 treatment on GCG gene expression in intestinal GLUTag cells at 2mM glucose. * $p < 0.05$ compared to untreated control. Δ $p < 0.05$ compared to vehicle control.

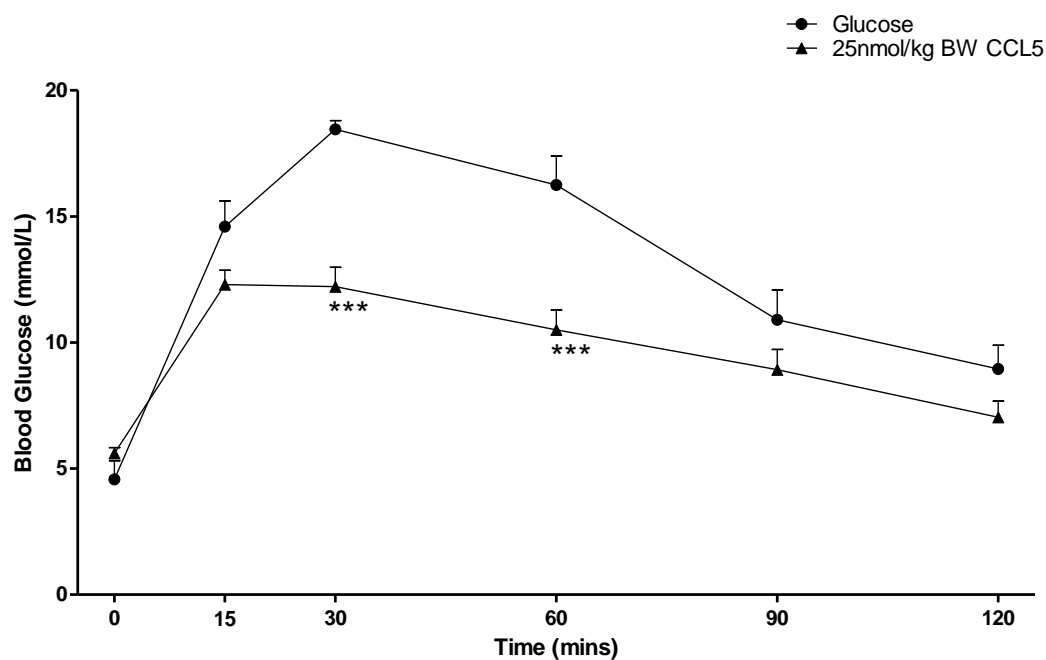
B.



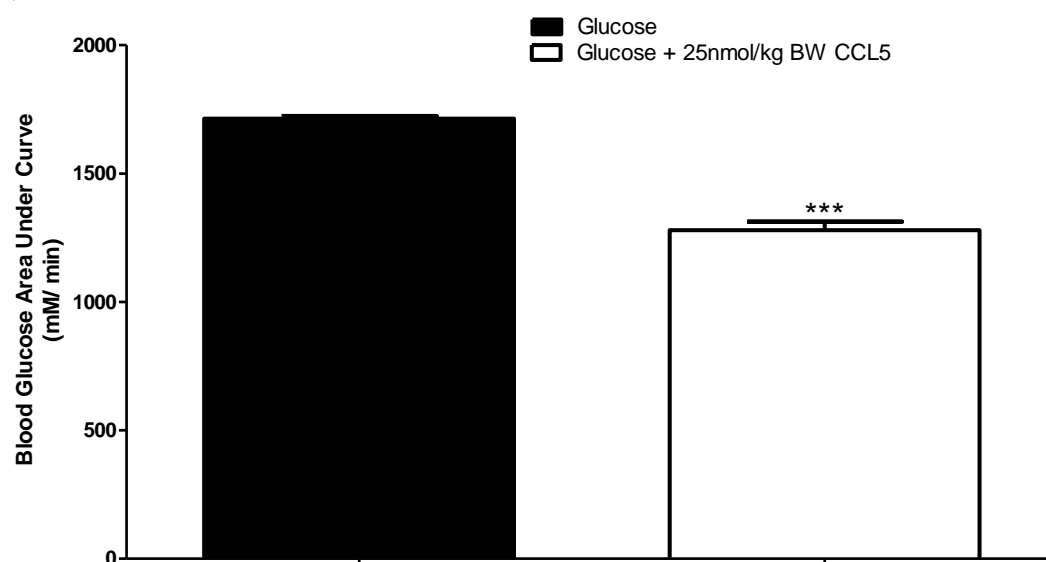
Effects of CCL5 treatment on PC1 gene expression in intestinal GLUTag cells at 2mM glucose. * $p < 0.05$ compared to untreated control. Δ $p < 0.05$ compared to vehicle control.

Figure 3.17: Acute effects of IP administration of CCL5 on blood glucose in male Swiss TO mice

A.



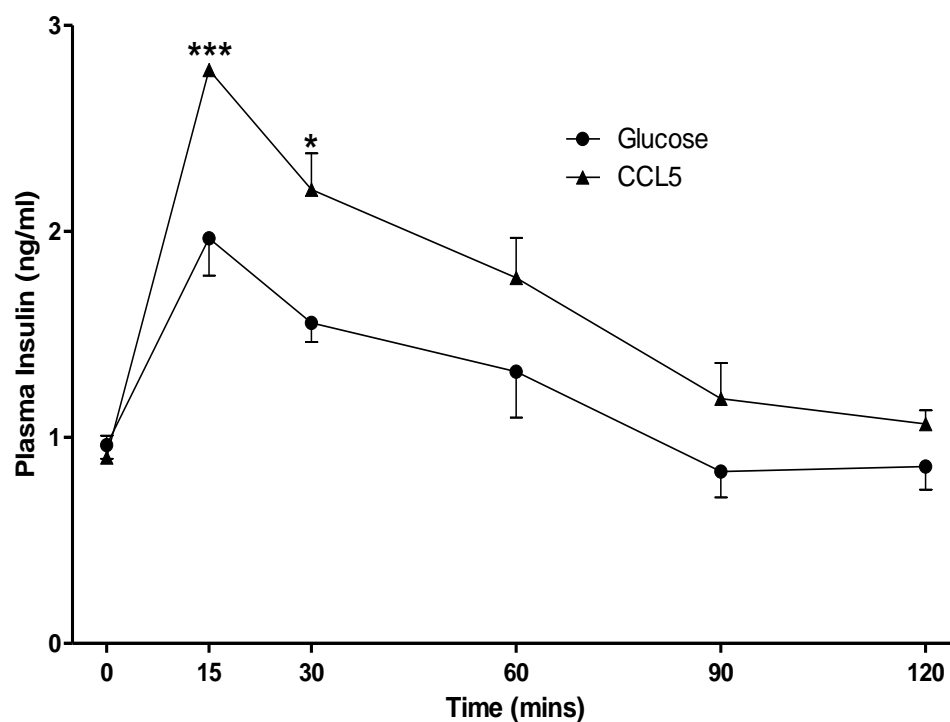
B.



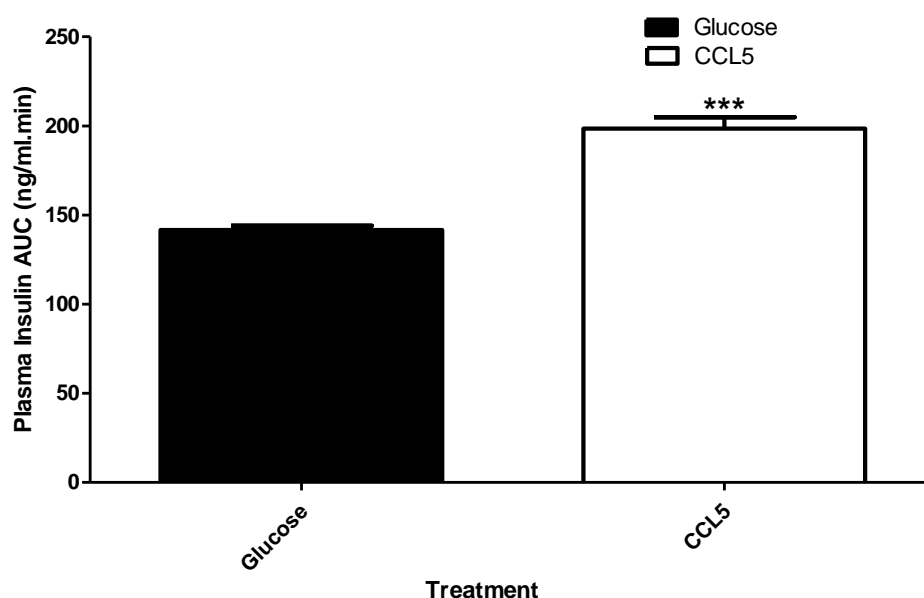
Glucose (18mmol/kg BW) or glucose in combination with CCL5 (25nmol/kg BW) were administered via IP injection to male Swiss TO mice (n=6). (A) Blood glucose (B) AUC of A. *** p<0.001 compared to glucose treatment alone.

Figure 3.18: Acute effects of IP administration of CCL5 on plasma insulin in male Swiss TO mice

A.



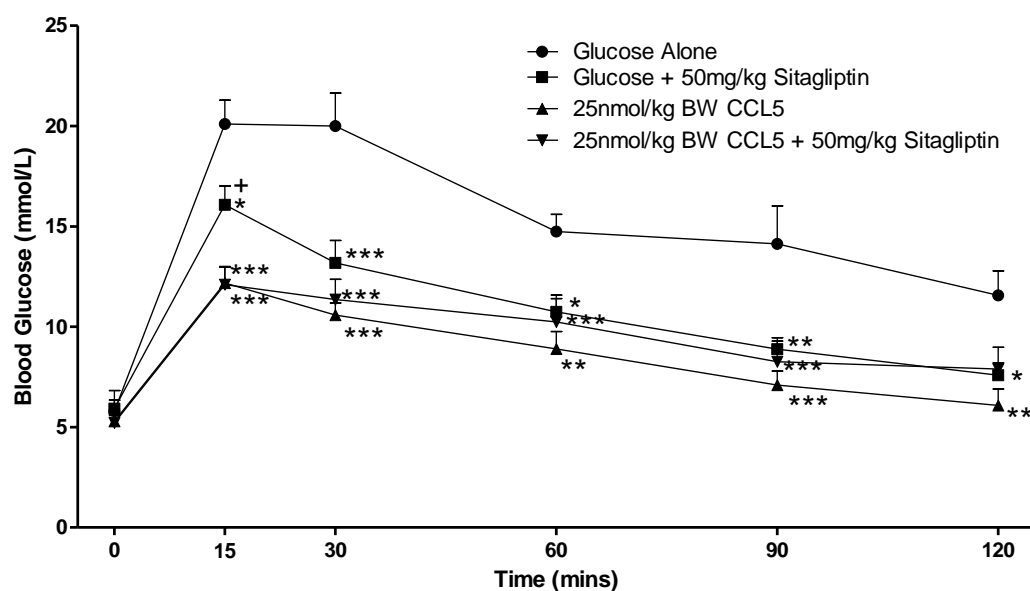
B.



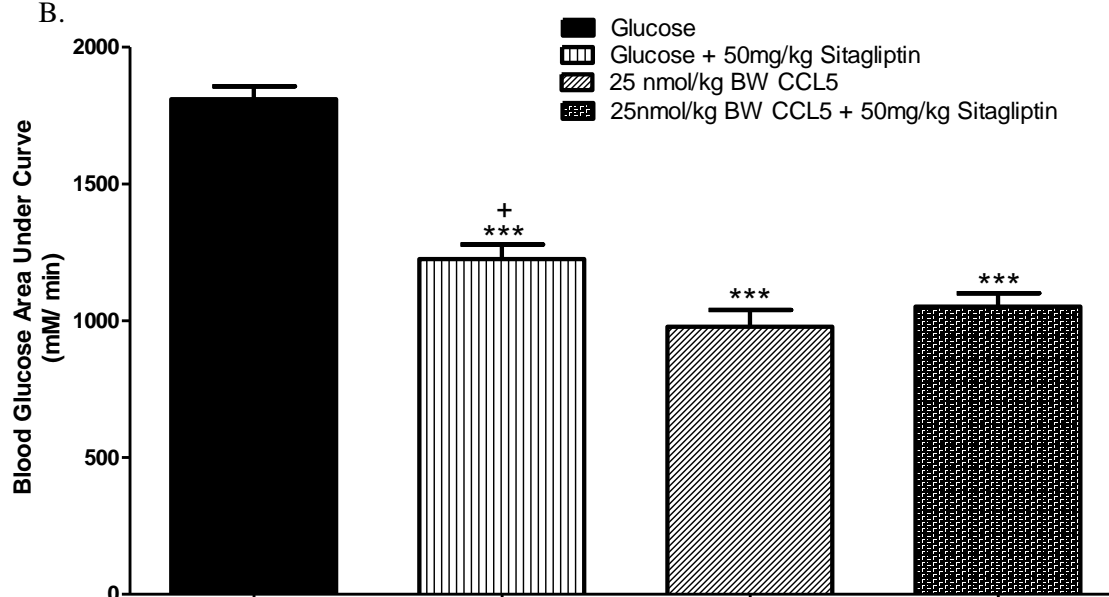
Glucose (18mmol/kg BW) or glucose in combination with CCL5 (25nmol/kg BW) were administered via IP injection to male Swiss TO mice (n=6). (A) Plasma insulin (B) AUC of A. * $p < 0.05$ and *** $p < 0.001$ compared to glucose treatment alone.

Figure 3.19: Acute effects of oral administration of CCL5 on blood glucose in male Swiss TO mice

A.



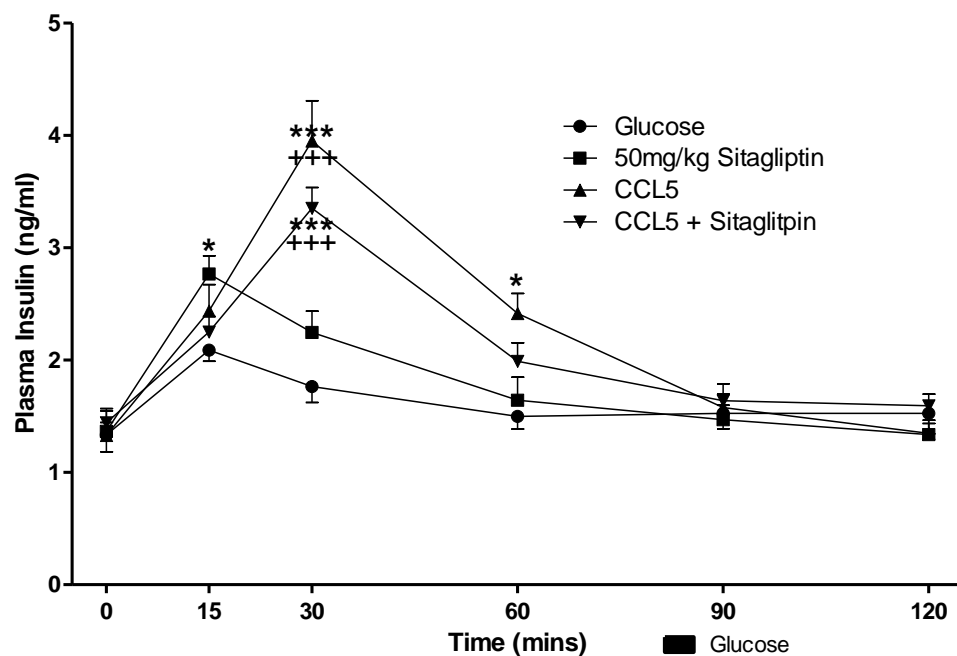
B.



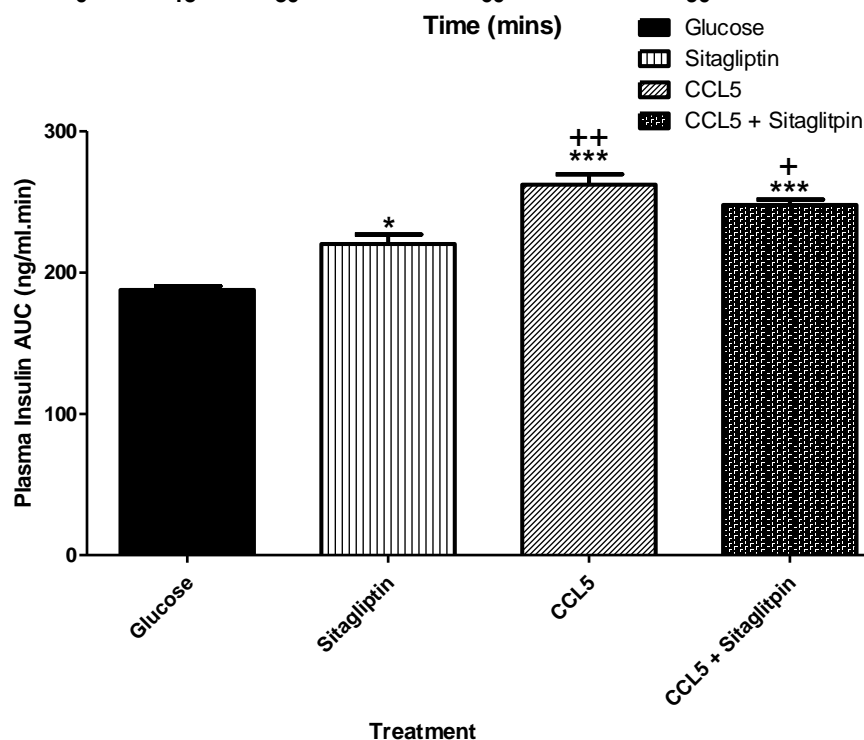
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with CCL5 (25nmol/kg BW) or glucose in combination with CCL5 and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Blood glucose (B) AUC of A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose treatment alone. + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ compared to Sitagliptin treatment alone.

Figure 3.20: Acute effects of oral administration of CCL5 on plasma insulin in male Swiss TO mice

A.



B.



Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with CCL5 (25nmol/kg BW) or glucose in combination with CCL5 and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin (B) AUC of A. * p<0.05, ** p<0.01 and *** p<0.001 compared to glucose treatment alone. + p<0.05, ++ p<0.01 and +++ p<0.001 compared to Sitagliptin treatment alone.

Chapter 4

Examining the role of trace metal ions on
GPR39 activation and incretin hormone
and insulin secretion *in vitro* and *in vivo*

4.1: Overview of results

Originally thought to be the obestatin receptor, the rhodopsin like GPR39 can be activated by trace metals. While originally found in the brain, the presence of GPR39 has been confirmed in a range of peripheral tissues such as throughout the gastrointestinal tract and the pancreas. Previous work has shown that GPR39 activation through trace metals can increase insulin secretion while some of these trace metals increase insulin secretion through other pathways as well. There are very few studies looking at the activation of GPR39 on incretin and PYY hormone secretion, however one bioavailable GPR39 agonist has been developed. The current study has identified the effects of GPR39 activation by trace metals CuCl_2 , NiSO_4 and ZnCl_2 on hormone secretion from GLUTag, pGIPneo STC-1 and BRIN-BD11 cells. Cytotoxicity was determined by Alamar Blue and MTT assay while membrane integrity was ascertained by LDH assay. Immunohistochemistry, qPCR determined the expression profile of GPR39 in the cell lines tested as well as in *ex vivo* intestinal tissue. This was further confirmed using qPCR. *In vivo* effects of ZnCl_2 and CuCl_2 ($50\mu\text{mol/kg BW}$) were determined in fasted male Swiss TO mice ($n=6$) both alone and in combination with Sitagliptin (50mg/kg BW).

In GLP-1 secreting GLUTag cells, CuCl_2 (31.25 to $500\mu\text{M}$) resulted in a 2.4 to 2.9-fold increase in GLP-1 secretion ($p<0.05$) in 2mM glucose while ZnCl_2 and NiSO_4 ($125\mu\text{M}$) reduced the expression of GPR39 3.1 and 1.3-fold respectively ($p<0.01$). CuCl_2 ($125\mu\text{M}$) treatment resulted in a 1.4-fold increase in GIP secretion from pGIPneo STC-1 cells ($p<0.01$) while NiSO_4 demonstrated a 1.3-fold increase ($125\mu\text{M}$, $p<0.01$). ZnCl_2 ($125\mu\text{M}$) also demonstrated the ability to increase GIP secretion *in vitro* with a 1.3-fold increase being observed ($p<0.01$).

Gene expression studies using qPCR demonstrated a 1.3-fold decrease in GPR39 expression in the small intestine, of HFF mice ($p<0.05$). Double immunohistochemistry confirmed the presence of the GPR39 receptor in both L and K-cells with co-localisation found between GPR39 and GIP as well as PYY. No GLP-1 secreting L-cells were found to have GPR39 present. Oral administration of GPR39 agonists in fasted male Swiss TO mice resulted in a reduction of blood glucose and increased insulin secretion. CuCl_2 demonstrated an overall ability to decrease blood glucose 25.3% ($p<0.05$) while ZnCl_2 decreased blood glucose 36.5% ($p<0.001$). These agonists increased insulin secretion 49.1% ($p<0.001$) and 86.4% ($p<0.001$). The DPP-IV inhibitor Sitagliptin decreased blood glucose 13.0% while combination of GPR39 agonists with Sitagliptin resulted in further reductions of blood glucose as well as further increases in insulin secretion. CuCl_2 in combination with Sitagliptin reduced blood glucose 46.3% ($p<0.001$) with a further reduction of 36.2% ($p<0.001$) compared to Sitagliptin alone while insulin secretion was increased 63.5% ($p<0.001$). ZnCl_2 administration in combination with Sitagliptin reduced blood glucose 48.5% ($p<0.001$) while this reduction was 39.6% ($p<0.001$). ZnCl_2 in combination increased insulin secretion 85.3% ($p<0.001$).

GPR39 agonists had no effect on feeding although CuCl₂ in combination resulted in a 15% decrease in appetite after 3 hrs.

This study demonstrates a role for GPR39 in the regulation of intestinal enteroendocrine cells and suggests that the activation of GPR39 by trace metals may be used as a potential therapy for Type 2 Diabetes in the future.

4.2: Introduction

GPR39 was first identified along with GPR38 as a ghrelin family receptor with 52% amino acid sequence homology to the human ghrelin receptor GHS-R1a (McKee *et al.* 1997). The 230kb GPR39 gene encodes a 453 amino acid protein. Using FISH, the authors of this work were able to map the GPR39 coding gene to chromosome 2q21-q22 in humans. GPR39 was found to be expressed in the brain as well as other peripheral tissues such as the intestine, stomach and pancreas. Like GPR38 (Feighner *et al.* 1999) and GHS-R (Howard *et al.* 1996), GPR39 has two variants with the full length seven transmembrane domain GPR39-1a and the shorter five transmembrane domain GPR39-1b (Egerod *et al.* 2007a). This work identified that GPR39 has a 200kb intron in a similar position to both GPR38 and GHS-R which separates the last 2 transmembrane domains from the first 5. It was also discovered that the antisense gene for GPR39 also coded for Ly-6/PLAUR domain containing 1 (LYPD) gene which is transcribed along with the truncated form of the GPR39 gene.

Like other receptors in the ghrelin family, GPR39 has high levels of activity without being activated by an agonist (Popovics, Stewart 2011) but the first identified agonist of GPR39 was obestatin (Zhang *et al.* 2005b). This study identified a new hormone which was post-translationally processed from the preproghrelin protein and named it obestatin. This name was due to the appetite reducing effects identified in rats treated with obestatin. Radiolabelled obestatin was found to bind to an orphan GPCR. Transfection of Chinese Hamster ovary cells with various cDNAs of orphan GPCRs were used to test which GPCR obestatin binds to. It was found that GPR39 bound the radiolabelled obestatin suggesting it is the obestatin receptor. This work was further supported by work in GPR39 knockout mice which had quicker gastric emptying, increased body weight, increased body fat composition and increased cholesterol (Moechars *et al.* 2006).

However, the identification of GPR39 as the obestatin receptor and the appetite reducing effects of obestatin remain controversial. A response to the original research into GPR39 and obestatin disputed the authors' findings (Chartrel *et al.* 2007) and the authors were unable to reproduce their initial *in vitro* results (Zhang *et al.* 2007). Further studies using GPR39 knockout mice found no difference in body weight and food intake when compared to wild type mice regardless of gender

(Tremblay *et al.* 2007). There was also no significant effect on plasma insulin or blood glucose levels in 24-week old mice. Injection of obestatin into the mice produced no effect on feeding over 24 hrs. Treatment of cells transfected with a cDNA clone for GPR39 with human or mouse obestatin had no effect on transcriptional activity of cells transfected with the clone suggesting that obestatin does not activate GPR39. This was further supported by work which showed that obestatin treatment of cells transfected with GPR39 cDNA did not increase cAMP or intracellular calcium (Lauwers *et al.* 2006).

As the search for endogenous GPR39 agonists continued, the receptors' expression in the stomach and the small intestine suggested that meal ions may be potential agonists. This led to the identification of ZnCl_2 as a GPR39 agonist (Holst *et al.* 2004). Further work also looking at obestatin, identified an increase in downstream signalling events upon treatment with Zn^{2+} while this effect was not seen with obestatin (Holst *et al.* 2007b). Radiolabelled obestatin also did not bind to GPR39 expressing cells. This was supported by later work that identified that treatment of cells transfected with a clone of human GPR39 cDNA with Zn^{2+} resulted in increased intracellular calcium (Lauwers *et al.* 2006). Phospholipase C (PLC) inhibitor stopped the agonistic effects of Zn^{2+} demonstrating that GPR39 can work through the G_{aq} pathway (Yasuda *et al.* 2007). The receptor has also been shown to increase serum response element (SRE)-mediated transcription which suggests that GPR39 can also work through $G_{\alpha 12/13}$ (Holst *et al.* 2004).

GPR39 null mice have been shown to have increased anxiety and demonstrate depression-like behaviour and GPR39 has been identified as a possible target in the treatment of depression (Młyniec *et al.* 2015). GPR39 was also found to be present in the pancreatic β -cells but not in the glucagon releasing α -cells, the somatostatin producing δ -cells or the pancreatic polypeptide PP cells (Holst *et al.* 2009a). Interestingly the same study looked at plasma glucagon levels in wild type versus GPR39 knockout mice and found glucagon levels were decreased in knockout mice, suggesting a role for GPR39 in glucagon secretion. Blood glucose was increased in GPR39 null female mice and an increase in insulin was also observed. GPR39 expression was high in fat from pregnant females (Fontenot, DeVente & Seidel 2007) and is found to be upregulated in adipose tissue in both fasting and streptozotocin induced diabetic rats (Egerod *et al.* 2007a) and GPR39 may play a protective role for β -cells in mice (Egerod *et al.* 2011). In humans, the expression of GPR39 has been found to be down regulated in the adipose tissue of obese patients with Type 2 Diabetes (Catalan *et al.* 2007). All of this research supports the concept that GPR39 may play an important role in glucose homeostasis.

One such study used an obesity induced diabetic mice model where mice fed a high fat diet had decreased GPR39 expression in adipose tissue. (Verhulst *et al.* 2011). However the majority of these studies have explored the knocking out of the GPR39 receptor and have not induced its

activation by an agonist. The effects of various trace metals on GPR39 activation and its role in glucose homeostasis was therefore investigated previously by our research group (Moran *et al.* 2016b). Trace metals Zn^{2+} , Cu^{2+} , Ni^{2+} and Co^{2+} were all found to increase insulin secretion *in vitro*. Both intracellular calcium and cAMP were increased and oral administration of GPR39 agonists resulted in reduced blood glucose and increased insulin secretion with the exception of Co^{2+} .

It is known that GPR39 is expressed throughout the gastrointestinal tract and may play a role in its regulation. In fact zinc tablets are currently a treatment for diarrhoea (Nasrin *et al.* 2005) thus giving this idea credence. However the majority of these studies have looked into obestatin and have not looked into the activation of GPR39 by trace metal agonists. One study has developed the first bioavailable oral GPR39 agonist which increased GLP-1 secretion in the mouse enteroendocrine STC-1 cell line (Peukert *et al.* 2014). As our previous work has determined that oral administration of GPR39 agonists *in vivo* reduces blood glucose and increases insulin secretion, we propose that this is through increased gastrointestinal hormone secretion. The effects of transition metals Cu^{2+} , Ni^{2+} and Zn^{2+} were tested in the GLP-1 secreting GLUTag cell line, GIP secreting pGIPneo STC-1 cells and PYY secreting STC-1 cells. The effects of these agonists on receptor and hormone expression was also investigated along with their effects on cell viability. Both Zn^{2+} and Cu^{2+} were then tested *in vivo* in male Swiss TO mice.

4.3: Materials and methods

4.3.1: Materials

Copper chloride (CuCl_2), nickel sulphate (NiSO_4) and zinc chloride (ZnCl_2), were purchased from Sigma-Aldrich (Poole, Dorset, UK). Multi species GLP-1 total and rat/mouse GIP total ELISA were purchased from Merck Millipore (Watford, UK).

4.3.2: Acute hormone secretion from intestinal cell lines

GLUTag or pGIPneo STC-1 cells were seeded into 24-well plates with 150000 cells per well which were incubated overnight in 1ml of culture medium (as described previously) at 37°C and 5% CO_2 . To ensure that cells grew in monolayers, they were pipetted up and down vigorously to ensure proper mixing before seeding. After overnight incubation and preincubation with 1.1mM glucose for 40 mins, acute incubations were carried out at 37°C 5% CO_2 for 2 hrs in 2mM glucose in KRBB buffer supplemented with 1.95 μM to 500 μM concentrations of CuCl_2 , NiSO_4 and ZnCl_2 . After incubations, supernatants (950 μl) were collected and frozen at -20°C until ELISA was carried out.

4.3.3: Acute insulin secretion from pancreatic BRIN-BD11 cells

Cells were seeded in 24-well with 150000 BRIN-BD11 cells per well and incubated overnight in 1ml of RPMI 1640 media at 37°C and 5% CO₂. Following this incubation, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 1.95µM to 500µM concentrations of CuCl₂, NiSO₄ and ZnCl₂ with 10mM alanine as a positive control. After incubations, supernatants (950µl) were collected and frozen at -20°C until radioimmunoassay was carried out.

4.3.4: Immunofluorescence staining in GLUTag, pGIPneo STC-1, STC-1 cells and lean and HFF NIH Swiss mouse small intestine

GLUTag, pGIPneo STC-1 and STC-1 cells were seeded at a density of 40000 cells on to polylysine coated slides overnight at 37°C in an atmosphere of 95% air and 5% CO₂ as described in Chapter 2, section 2.1. Swiss TO mice were anaesthetised by isoflurane and killed by cervical dislocation. Pancreas and intestine was excised as described in Chapter 2, section 2.6.3. Immunohistochemistry was carried out by incubating with rabbit polyclonal anti-GPR39 and goat polyclonal anti-GLP-1, goat polyclonal anti-GIP or goat polyclonal anti-PYY with the dilutions outlined in Chapter 2, Table 2.1.

4.3.5: Acute effects of CuCl₂, NiSO₄ and ZnCl₂ on cell toxicity

Cells were seeded in 96-well plates with 40000 cells per well of BRIN-BD11, GLUTag or pGIPneo STC-1 cells and incubated overnight in cell culture media (as described in Chapter 2, section 2.1) in an atmosphere of 5% CO₂ at 37°C. After 20hrs, BRIN-BD11 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 1.95µM to 500µM concentrations of GPR39 agonist with 1mM H₂O₂ as a cytotoxic control (n=3). After 48hrs, GLUTag and pGIPneo STC-1 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 2 hrs in 2mM in KRBB buffer supplemented with 1.95µM to 500µM concentrations of GPR39 agonists with 1mM H₂O₂ as a cytotoxic control (n=3). After incubation, supernatant was decanted, cells were washed with HBSS and 0.5mg/ml MTT was added for 2 hrs. Following MTT incubation, supernatant was discarded and DMSO was added to solubilise crystals. Optical density was subsequently measured at 570nm and 630nm using the Flexstation 3 (Molecular Devices, CA, USA). LDH assay was carried out as described previously in Chapter 2, section 2.4.2.

4.3.6: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Chapter 2, Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH.

4.3.7: Acute *in vivo* glucose tolerance tests

Male Swiss TO mice were housed as described in Chapter 2, section 2.7. The effects of GPR39 agonists CuCl₂ and ZnCl₂ (50µmol/kg BW), as well as in combination with Sitagliptin Phosphate (50mg/kg BW) in lean male Swiss TO mice were investigated. Before test compounds were administered mice were fasted for 18 hrs and blood subsequently collected (t=0). Test compounds were administered orally in glucose (18mmol/kg BW), and blood was collected at 15, 30, 60, 90 and 120 mins along with blood glucose measurements. Collected blood was centrifuged at 13200rpm for 5 mins at 4°C using a Beckman centrifuge (Beckman Instruments, UK). Plasma was stored at -20°C until hormone measurements by RIA or ELISA as described in Chapter 2, section 2.2 and 2.3.

4.4: Results

4.4.1: Effects of GPR39 agonists on GLP-1 secretion from intestinal GLUTag cells

GLUTag cells were treated for 2 hrs with GPR39 agonists (31.25-125µM) in 2mM glucose. Treatment of GLUTag cells with CuCl₂ (31.3 to 125µM) resulted in a 2.4 to 2.9-fold increase in GLP-1 secretion (p<0.05) in 2 mM glucose (Figure 4.1A). Treatment of GLUTag cells with NiSO₄ (31.3 to 125µM) or ZnCl₂ (31.3 to 125µM) had no effect on GLP-1 secretion (Figure 4.2A and 4.3A).

4.4.2: Effects of GPR39 agonists on GIP secretion from intestinal pGIPneo STC-1 cells

The effects of GPR39 agonist treatment on pGIPneo STC-1 cells were investigated by measuring GIP secretion via ELISA. All agonists tested demonstrated an increase in GIP secretion. CuCl_2 (31.3-125 μM) had the greatest effect on GIP secretion with a 1.3 to 1.4-fold increase ($p < 0.05$ - $p < 0.01$) in secretion (Figure 4.4A). NiSO_4 (31.3-125 μM) increased GIP 1.21 to 1.33-fold ($p < 0.05$ - $p < 0.01$) compared to basal control (Figure 4.5A). ZnCl_2 (31.3-125 μM) increased GIP secretion 1.2 to 1.3-fold from pGIPneo STC-1 cells, compared to basal levels ($p < 0.01$) (Figure 4.6A).

4.4.3: Effects of GPR39 agonists on insulin secretion from pancreatic BRIN-BD11 cells

The effects of transition metal GPR39 agonists CuCl_2 , NiSO_4 and ZnCl_2 on insulin secretion from pancreatic BRIN-BD11 cells were tested in both 5.6mM and 16.7mM glucose (Figures 4.7-4.12). In normoglycaemic conditions, CuCl_2 demonstrated the best insulinitropic ability followed by ZnCl_2 then NiSO_4 . Treatment of BRIN-BD11 cells with CuCl_2 resulted in a 1.2 to 2.8-fold increase in insulin secretion (15.6-500 μM , $p < 0.001$) when compared to secretion levels from cells treated with 5.6mM glucose (Figure 4.7A). BRIN-BD11 cells treated with GPR39 agonist ZnCl_2 exhibited a 1.2 to 2.4-fold increase (15.6-500 μM , $p < 0.01$ - $p < 0.001$) in insulin secretion compared to basal secretion levels (Figure 4.11A). NiSO_4 treatment of BRIN-BD11 cells produced a 1.4 to 2.4-fold increase (31.25-500 μM , $p < 0.001$) in insulin secretion (Figure 4.9A).

ZnCl_2 was the GPR39 agonist which demonstrated the greatest insulinitropic effect of acute treatment of BRIN-BD11 cells in hyperglycaemic conditions (Figure 4.12A). Insulin secretion was increased 1.2 to 2.2-fold (15.6-500 μM , $p < 0.001$), compared to 16.7mM glucose. CuCl_2 had a slightly lower insulinitropic effect than ZnCl_2 while NiSO_4 provided the lowest effect on insulin secretion as demonstrated in 5.6mM glucose. CuCl_2 (Figure 4.8A) increased insulin secretion 1.2 to 2.2-fold (15.6-500 μM , $p < 0.01$ - $p < 0.001$) while NiSO_4 (Figure 4.10A) exhibited a 1.4 to 2.0-fold increase (31.3-500 μM , $p < 0.05$ - $p < 0.001$) in insulin secretion, compared to basal levels.

In normoglycaemic conditions, CuCl_2 , was also the most potent agonist tested with an EC_{50} value of $66.3 \pm 0.9\mu\text{M}$. Despite being the worst performing agonist in relation to insulin secretion, NiSO_4 was more potent than ZnCl_2 with EC_{50} values of $104.5 \pm 1\mu\text{M}$ and $142.2 \pm 1\mu\text{M}$ respectively (Figure 4.13A).

In hyperglycaemic conditions, NiSO_4 , was determined to be the most potent with an EC_{50} value of $47.4 \pm 1\mu\text{M}$. This was followed by CuCl_2 , ($51.9 \pm 1.2\mu\text{M}$) and ZnCl_2 ($235.2 \pm 1.1\mu\text{M}$) (Figure 4.13B).

4.4.4: Effects of GPR39 activation on cell viability in BRIN BD11, GLUTag and pGIPneo STC-1 cells

MTT assay was used to assess the effects of GPR39 agonists on GLUTag cells. CuCl_2 (2-500 μM) had no effect on GLUTag cell viability (Figure 4.1B), however NiSO_4 (Figure 4.2B) reduced cell viability 1.5-fold at the highest concentration tested (500 μM , $p < 0.05$). Due to the reduced cell viability of GLUTag cells treated with NiSO_4 at the highest concentration tested, a membrane integrity assay was carried out using LDH assay. This assay showed that NiSO_4 (250, 125 and 7.8 μM , $p < 0.05$ - $p < 0.001$) and ZnCl_2 (250, 62.5, 31.25 and 3.9 μM , $p < 0.05$ - $p < 0.001$) increased membrane integrity in GLUTag cells (Figures 4.2C and 4.3C).

Treatment of pGIPneo STC-1 cells with CuCl_2 resulted in a 1.2 to 1.3-fold increase (Figure 4.4B) in cell viability (1.95-125 μM , $p < 0.05$ - $p < 0.01$). Unlike in GLUTag cells where viability was reduced, treatment of pGIPneo STC-1 cells with NiSO_4 resulted in a 1.1 to 1.2-fold increase (Figure 4.5B) in cell viability (7.8, 31.3 and 500 μM , $p < 0.05$). ZnCl_2 reduced cell viability 1.7-fold (Figure 4.6B) at the highest concentration tested (500 μM , $p < 0.01$).

The effects of GPR39 agonists on the cell viability of BRIN-BD11 cells was tested using Alamar Blue assay. All agonists (2-500 μM) had no toxic effects on BRIN-BD11 cells (Figures 4.7B, 4.8B, 4.9B, 4.10B, 4.11B and 4.12B). In order to assess the relevancy of MTT assay for GPR39 agonists, an MTT assay was carried out for NiSO_4 and ZnCl_2 in BRIN BD11 cells (Figures 4.9C, 4.10C, 4.11C and 4.12C). As seen with Alamar Blue assay, MTT assay determined that GPR39 agonists have no cytotoxic effects in BRIN BD11 cells.

4.4.5: Distribution of GPR39, GLP-1, GIP and PYY in intestinal cell lines and lean and HFF NIH Swiss mouse small intestine

In order to determine the effects of GPR39 both *in vitro* and *in vivo*, suitable models must be selected. The expression of GPR39 in the endocrine cells of the intestine was determined through double immunohistochemistry in both lean and HFF diet mice as well as GLUTag, pGIPneo STC-1 and STC-1 cells.

In GLUTag cells, GPR39 and GLP-1 demonstrated areas of co-localisation (Figure 4.14D). However in lean and HFF small intestine from NIH Swiss mice, no GLP-1 secreting L cells were found to have GPR39 present (Figure 4.15C and 4.16C).

Double immunohistochemistry determined that both GIP and GPR39 were present in pGIPneo STC-1 cells (Figure 4.18). The presence of GPR39 in GIP secreting intestinal K-cells was confirmed

in both lean (Figure 4.19C) and HFF (Figure 4.20C) mouse small intestine as areas of co-localisation between GIP and GPR39 were observed (Figures 4.19D and 4.20D).

The expression of GPR39 along with PYY was investigated in STC-1 cells (Figure 4.21). Double immunohistochemistry confirmed the presence of GPR39 in PYY secreting L cells as co-localisation was demonstrated (Figure 4.21D). The presence of GPR39 in PYY secreting L cells was also confirmed in lean and HFF mouse small intestine (Figure 4.22D and 4.23D) with areas of co-localisation between GPR39 and PYY being observed.

The differences in GPR39 gene expression in lean and HFF NIH Swiss mouse small intestine were determined using qPCR (Figure 4.17). NIH Swiss mice fed a diet rich in a HFF diet results in a 1.3-fold reduction ($p<0.05$) in intestinal GPR39 gene expression.

4.4.6: Effects of GPR39 agonists on proglucagon gene expression in intestinal GLUTag cells

In order to determine the effects of GPR39 agonists on proglucagon gene expression in intestinal L cells, GLUTag cells were treated with GPR39 agonists at a concentration of 125 μ M (Figure 4.24). Treatment with GPR39 agonists NiSO₄ and ZnCl₂ reduced GPR39 gene expression 1.3 and 3.1-fold respectively compared to untreated cells ($p<0.01$). When compared to 2mM glucose, there was a 2.9-fold decrease in proglucagon expression in GLUTag cells treated with ZnCl₂ ($p<0.05$).

4.4.7: Acute effects of GPR39 agonists on incretin hormones in male Swiss TO mice

Male Swiss TO mice were administered GPR39 agonists orally so that the effect of these agonists on the secretion of incretin hormones could be assessed. Administration of CuCl₂ (Figure 4.25A) increased GLP-1 secretion 2.0 ($p<0.001$) and 3.3-fold ($p<0.001$) 15 and 30 mins post administration respectively. ZnCl₂ (Figure 4.25A) increased GLP-1 secretion 2.9-fold ($p<0.001$) 30 mins post administration. Overall CuCl₂ increased GLP-1 secretion 106.7% ($p<0.001$) while ZnCl₂ increased GLP-1 secretion 96.5% ($p<0.001$) as measured by AUC (Figure 4.25B).

Both CuCl₂ and ZnCl₂ (Figure 4.26A) increased GIP secretion with CuCl₂ increasing secretion 2.6-fold ($p<0.001$) 15 mins post administration. ZnCl₂ increased GIP secretion 2.2 ($p<0.01$) and 4.1-fold ($p<0.001$) 15 and 30 mins post administration respectively. ZnCl₂ had an overall increase of 165.1% ($p<0.001$) while CuCl₂ increased GIP secretion 72.0% ($p<0.001$) as measured by AUC (Figure 4.26B). ZnCl₂ also increased GIP secretion compared to CuCl₂ with an increase of 54.1% ($p<0.05$) being observed (Figure 4.26B).

4.4.8: Acute effects of GPR39 agonists on blood glucose and insulin in male Swiss TO mice

Oral administration of GPR39 agonist CuCl_2 (50 $\mu\text{mol/kg}$ BW) in Swiss TO mice reduced blood glucose 41.2% 30 mins after dosage ($p<0.01$) and this effect persisted until 60 mins with a 26.8% decrease observed ($p<0.05$, Figure 4.27A). Sitagliptin alone resulted in a reduction of 26.6% ($p<0.01$) 60 mins post administration (Figure 4.27A). Sitagliptin significantly reduced blood glucose from 15 to 30 mins post administration with reductions of 22.3% ($p<0.05$) and 23.7% ($p<0.01$).

Combination of CuCl_2 and Sitagliptin had the greatest effect on blood glucose with a reduction seen from 15 to 60 mins (Figure 4.27A). After 15 mins, blood glucose was reduced 45.0% ($p<0.001$) compared to glucose while there was a 35.1% reduction compared to CuCl_2 alone ($p<0.001$) and a 29.2% decrease compared to Sitagliptin ($p<0.05$). After 30 mins the reduction compared to glucose had increased to 55.2% ($p<0.001$) and was 34.9% after 60 mins ($p<0.001$).

The glucose lowering effects of combination treatment persisted for the duration of the test when compared to Sitagliptin alone. After 30 and 60 mins, blood glucose was reduced 41.2% ($p<0.001$) and 34.8% ($p<0.001$) respectively with reductions of 25.9% ($p<0.05$) and 30.2% ($p<0.05$) observed after 90 and 120 mins respectively. These reductions were confirmed with the AUC for each treatment (Figure 4.27B).

Overall Sitagliptin had the lowest effect on glycaemic excursion with a 13.0% reduction ($p<0.05$). CuCl_2 treatment alone resulted in a 25.3% overall reduction ($p<0.05$). Compared to Sitagliptin, CuCl_2 had a 14.1% reduction in reducing glycaemic excursion ($p<0.05$). Combination of both CuCl_2 and Sitagliptin had the greatest overall reducing effect on blood glucose with an overall reduction of 46.3% ($p<0.01$) compared to glucose being observed. Compared to Sitagliptin alone, this reduction was 38.3% ($p<0.001$) while compared to CuCl_2 alone it was 28.1% ($p<0.05$).

In terms of insulin secretion, oral administration of CuCl_2 increased secretion 4.3-fold 15 mins post administration (Figure 4.28A, $p<0.001$) compared to glucose administration. The insulinotropic effect of CuCl_2 was sustained 30 mins post administration with 2.0-fold increase being observed ($p<0.05$). The insulinotropic effect of CuCl_2 was delayed when administered in combination with Sitagliptin. Insulin secretion was increased 3.04-fold ($p<0.001$) 30 mins post administration. Sitagliptin treatment alone lead to an increase of 2.08-fold when compared to glucose alone ($p<0.01$). Overall there were no significant differences observed between Sitagliptin treatment alone and Sitagliptin in combination with CuCl_2 (Figure 4.28B).

Oral administration of ZnCl_2 reduced glycaemic excursion (Figure 4.27C) throughout the duration of the GTT with a reduction of 37.21% initially observed ($p<0.001$) followed by reductions of 41.7%, 30.9%, 57.6% and 49.7% at 30, 60, 90 and 120 mins respectively ($p<0.01$ - $p<0.001$).

Combination of ZnCl₂ also reduced blood glucose with reductions of 53.8%, 60.0%, 44.6%, 36.4% and 34.0% being observed from 15 to 120 mins ($p<0.01$ - $p<0.001$). Compared to Sitagliptin alone, ZnCl₂ reduced blood glucose 38.5%, 24.1%, 46.5% and 37.4% at 15, 30, 90 and 120 mins respectively ($p<0.01$ - $p<0.001$). ZnCl₂ in combination with Sitagliptin also lowered blood glucose compared to Sitagliptin alone with reductions of 54.8%, 47.9% and 33.9% at 15, 30 and 60 mins ($p<0.001$). Comparing combination treatment with ZnCl₂ alone resulted in reductions of 26.5% and 31.3% after 15 and 30 mins ($p<0.05$ - $p<0.01$) although blood glucose was increased 33.4% at 90 mins. The AUC for this (Figure 27D) showed that combination treatment had a greater overall effect than ZnCl₂ alone with a reduction of 19.0% observed ($p<0.05$). Compared to glucose combination treatment reduced glycaemic excursion 48.6% ($p<0.001$) while ZnCl₂ lowered glucose 36.5% ($p<0.001$). Compared to Sitagliptin this effect was 39.6% and 25.4% for combination treatment and ZnCl₂ alone respectively ($p<0.001$, $p<0.05$).

Oral Administration of ZnCl₂ lead to a 2.9-fold increase in insulin secretion 30 mins after administration (Figure 4.28C, $p<0.001$). ZnCl₂ in combination with Sitagliptin also demonstrated an insulintropic response with increases of 1.8 and 2.6-fold being observed 15 and 30 mins after administration respectively ($p<0.01$ - $p<0.001$). Overall, ZnCl₂ had no significant difference between ZnCl₂ in combination with Sitagliptin or Sitagliptin treatment alone (Figure 4.28D).

4.4.9: Acute effects of GPR39 agonists on feeding

In order to assess the acute effects of GPR39 agonists on feeding, male Swiss TO mice were trained to eat for 3 hrs daily (Figure 4.29). Treatment with CuCl₂ had no effect on feeding while CuCl₂ in combination with Sitagliptin Phosphate reduced feeding 14.4% ($p<0.05$) after 3 hrs (Figure 4.29A). ZnCl₂ had an initial reduction in feeding of 38.6% after 30 mins ($p<0.01$), however this effect was not sustained and there was no further appetite reducing effect observed (Figure 4.29B). ZnCl₂ in combination with Sitagliptin also had an initial appetite lowering effect with a reduction of 41.2% being observed after 30 mins ($p<0.01$). After 60 mins appetite was increased by 35.2% ($p<0.01$) when compared to ZnCl₂ treatment alone however this effect was also not sustained and no further changes in appetite were observed.

4.5 Discussion

There has been considerable interest into the activation of GPR39 by obestatin (Zhang *et al.* 2005b, Tremblay *et al.* 2007, Chartrel *et al.* 2007, Zhang *et al.* 2007, Holst *et al.* 2007b). This research has cast doubt not only on the activation of GPR39 by obestatin but also the appetite reducing effects of obestatin. Further work has identified zinc as a possible GPR39 agonist (Holst *et al.* 2004,

Lauwers *et al.* 2006, Holst *et al.* 2009a) and further work has implicated a role for GPR39 in the treatment of depression (Młyniec *et al.* 2015) and diabetes (Egerod *et al.* 2007a, Moran *et al.* 2016b). GPR39 has previously been shown to be present throughout the gastrointestinal tract (McKee *et al.* 1997) and has been shown to be present in the enteroendocrine cells *in vitro* (Peukert *et al.* 2014).

The present study assessed the *in vitro* effects of GPR39 agonists by transition metals on GLP-1, GIP and insulin secretion *in vitro*. The presence of GPR39 in the enteroendocrine cells of the small intestine has previously been confirmed (Peukert *et al.* 2014). In the GLP-1 secreting GLUTag cell line, both ZnCl₂ and NiSO₄ had no effect on GLP-1 secretion while CuCl₂ caused an increase in secreted GLP-1. Previously only one bioavailable GPR39 agonist has been developed that increases GLP-1 secretion (Peukert *et al.* 2014). It is therefore not a surprising finding that both Zn²⁺ and Ni²⁺ do not increase GLP-1 secretion while Cu²⁺ is the second GPR39 agonist which has been identified to increase GLP-1. There are currently no published studies which have looked at the effects of GPR39 activation on GIP secretion and this study determined that all 3 trace metals tested caused an increase in GIP secretion *in vitro*. This confirms previous *in vivo* work in our lab which was carried out in GLP-1 and GIP knockout mice which showed the incretin effect persisted in GLP-1 knockout mice but not GIP knockout mice (unpublished). In the insulin secreting BRIN BD11 cell line, CuCl₂ had the greatest insulin secretory effect followed by ZnCl₂ then NiSO₄ in normoglycaemic conditions. In hyperglycaemic conditions, ZnCl₂ provided the greatest insulinotropic effect followed by CuCl₂ then NiSO₄. This confirms the previous research showing that Zn²⁺ can increase insulin secretion (Lauwers *et al.* 2006, Holst *et al.* 2007b, Moran *et al.* 2016b) and the transition metals Cu²⁺ and Ni²⁺ increase insulin secretion *in vitro* (Moran *et al.* 2016b).

The localisation of GPR39 in the intestinal cell lines as well as lean and HFF mouse small intestine was confirmed using immunohistochemistry and qPCR. GPR39 has previously been shown to be present in the intestinal STC-1 cell line (Peukert *et al.* 2014) as well as throughout the gastrointestinal tract (McKee *et al.* 1997). The presence in STC-1 cells was confirmed in this study as well as the presence of GPR39 in GLUTag cells and pGIPneo STC-1 cells. GPR39 was also found to be present in PYY secreting L-cells but no co-localisation was found with GLP-1 secreting cells. Very few GPR39 agonists have been found to increase GLP-1 secretion and it may be due to the GPR39-1b isoform being present in GLP-1 secreting L-cells rather than the active GPR39-1a form although further work would need to be carried out to determine this. Feeding mice a high fat diet led to decreased expression in the small intestine which is an effect also seen in the adipose tissue of obese humans with Type 2 Diabetes (Catalan *et al.* 2007).

The treatment of intestinal cell lines with trace metal GPR39 agonists had some surprising results. Both NiSO₄ and ZnCl₂ reduced the cell viability of GLUTag cells at 500µM while this effect was

also seen for ZnCl₂ in pGIPneo STC-1 cells at the same concentration. It must be recognised that this is a high concentration. Many studies have shown that Zn²⁺ can be toxic to a range of cell lines (Toussaint, Nederbragt 1993, Walther *et al.* 2000, Daniels *et al.* 2004, Bozym *et al.* 2010) and some of these cells have shown toxicity at lower levels of zinc than used in this current study. Likewise Ni²⁺ has also shown to be toxic in mammalian cells (Skreb, Fischer 1984) and this may be due to the displacement of Fe²⁺ leading to a hypoxic response (Permenter, Lewis & Jackson 2011). Interestingly neither NiSO₄ nor ZnCl₂ break down the cell membrane as shown by LDH assay so the toxic effects seen appear to have a direct effect on cell metabolism.

Treatment of GLUTag cells with a non-toxic (125µM) dose of trace metal GPR39 may explain the secretory results. Treatment with both NiSO₄ and ZnCl₂ leads to significant down regulation of GPR39 while ZnCl₂ significantly downregulates GPR39. The agonist which increases GLP-1 secretion, CuCl₂, has no effect on GPR39 expression in GLUTag cells when compared to untreated cells or vehicle control. This suggests that the agonists which have no effect on secretion downregulate the receptor to prevent it from becoming activated leading to an increase in secretion. It may also lead to an increase in the expression of the GPR39-1b isoform although further work is needed to elucidate this.

In order to validate the *in vitro* findings discussed above, male Swiss TO mice were orally administered with either CuCl₂ or ZnCl₂ (50µmol/kg BW). Previous work in our lab has determined that IP administration of GPR39 trace metal agonists had no effect on blood glucose, however oral administration did reduce glucose in non-fasted mice (Moran *et al.* 2016b). The agonists were also administered as a combination treatment with Sitagliptin Phosphate (50 mg/kg BW) at a concentration used previously by our group (Gault, Lennox & Flatt 2015). Sitagliptin has been studied in combination with Metformin (Miller, St Onge & Accardi 2009) as well as other anti-diabetic drugs such as pioglitazone (Alba *et al.* 2009, Bailey, Green & Flatt 2010) and glimepiride (Ishii *et al.* 2014) and possible triple combination therapies have been postulated (Hirao *et al.* 2012). The glucose lowering effects previously seen in male non-fasted mice were confirmed in this study in fasted mice with both ZnCl₂ and CuCl₂ reducing blood glucose while also increasing insulin secretion thus confirming previous research which determined that Zn²⁺ had glucose lowering effects in obesity induced diabetic mice (Adachi *et al.* 2006). Combination of GPR39 agonists with Sitagliptin had further glucose lowering effects and this was significant compared to either agonist or Sitagliptin alone. However, the insulinotropic effects of this treatment did not show significant increases overall compared to GPR39 agonists or Sitagliptin treatment alone. CuCl₂ treatment alone did have a more rapid response in terms of insulin secretion than ZnCl₂, while the insulinotropic effect of ZnCl₂ was quicker and more sustained in combination with Sitagliptin than ZnCl₂ treatment alone. Previous work in our lab using incretin receptor knockout mice determined that GPR39

increases GIP secretion and it is likely that this decreased blood glucose was caused by an increase in GIP secretion along with delayed DPP-IV activity due to Sitagliptin treatment.

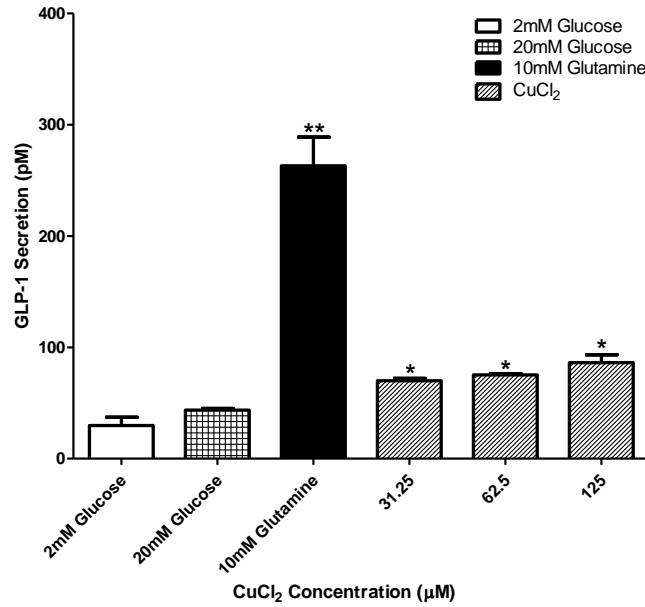
The acute effects of CuCl₂ and ZnCl₂ on GIP and GLP-1 secretion were also assessed. Previous work has shown that GPR39 agonists are likely to increase GIP secretion (Moran *et al.* 2016b). Both GPR39 agonists tested in this study demonstrated the ability to increase GIP secretion. Interestingly despite ZnCl₂ not showing an ability to increase GLP-1 secretion in GLUTag cells, both agonists were also able to increase GLP-1 secretion in male Swiss TO mice. Only one bioavailable GPR39 agonist has been shown to increase GLP-1 previously (Peukert *et al.* 2014) and this is the first study to directly show that GPR39 activation in the intestinal K cells can increase GIP secretion.

Male Swiss TO mice were trained to eat for 3 hrs daily. This protocol has found that trained mice complete the majority of their daily feeding within the first 2 hrs but need the third hr of eating to sustain their body weight (O'Harte *et al.* 1998b). GPR39 had previously been implicated in appetite reduction through obestatin (Zhang *et al.* 2005b) however this work could not be replicated *in vitro* and has been largely discredited. Administration of GPR39 agonists had no effect over the course of feeding in this model which backs up other studies have shown that GPR39 has no effect on appetite (Tremblay *et al.* 2007). CuCl₂ in combination with Sitagliptin did have a reducing effect on appetite and this may be due to it being the only GPR39 agonist tested that increased both GLP-1 secretion and GIP. Studies have shown that GLP-1 can decrease appetite with GIP having no effect (Turton *et al.* 1996, Tang-Christensen *et al.* 1996, Larsen *et al.* 2001, Chelikani, Haver & Reidelberger 2005, Edholm *et al.* 2010a).

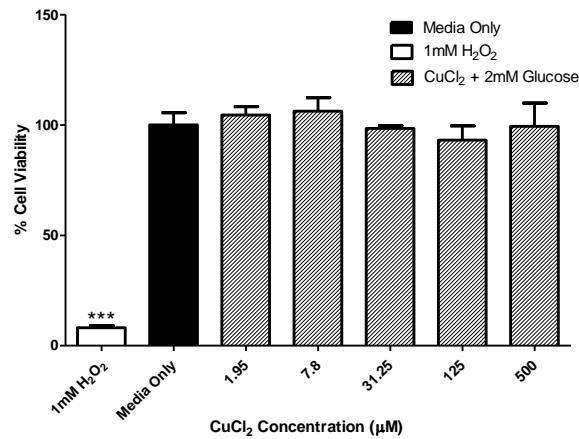
In conclusion, this study has determined that GPR39 agonists increase GLP-1, GIP and insulin secretion both *in vitro* and *in vivo*. GPR39 was found to be present in all of the intestinal cell lines tested, namely GIP-1 secreting cells (GLUTag), GIP secreting cells (pGIPneo STC-1) and PYY secreting cells (STC-1cells). Oral administration of GPR39 agonists reduces blood glucose and increases plasma insulin, GIP and GLP-1 in male Swiss TO mice but has no acute effects on appetite. GPR39 agonists work better in combination with Sitagliptin, a drug which has previously been used in combination therapies. These results implicate GPR39 in glucose homeostasis through incretin pathways and oral administration of GPR39 agonists may be a potential novel anti-diabetic therapy.

Figure 4.1: Effects of GPR39 agonist CuCl_2 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose

A.

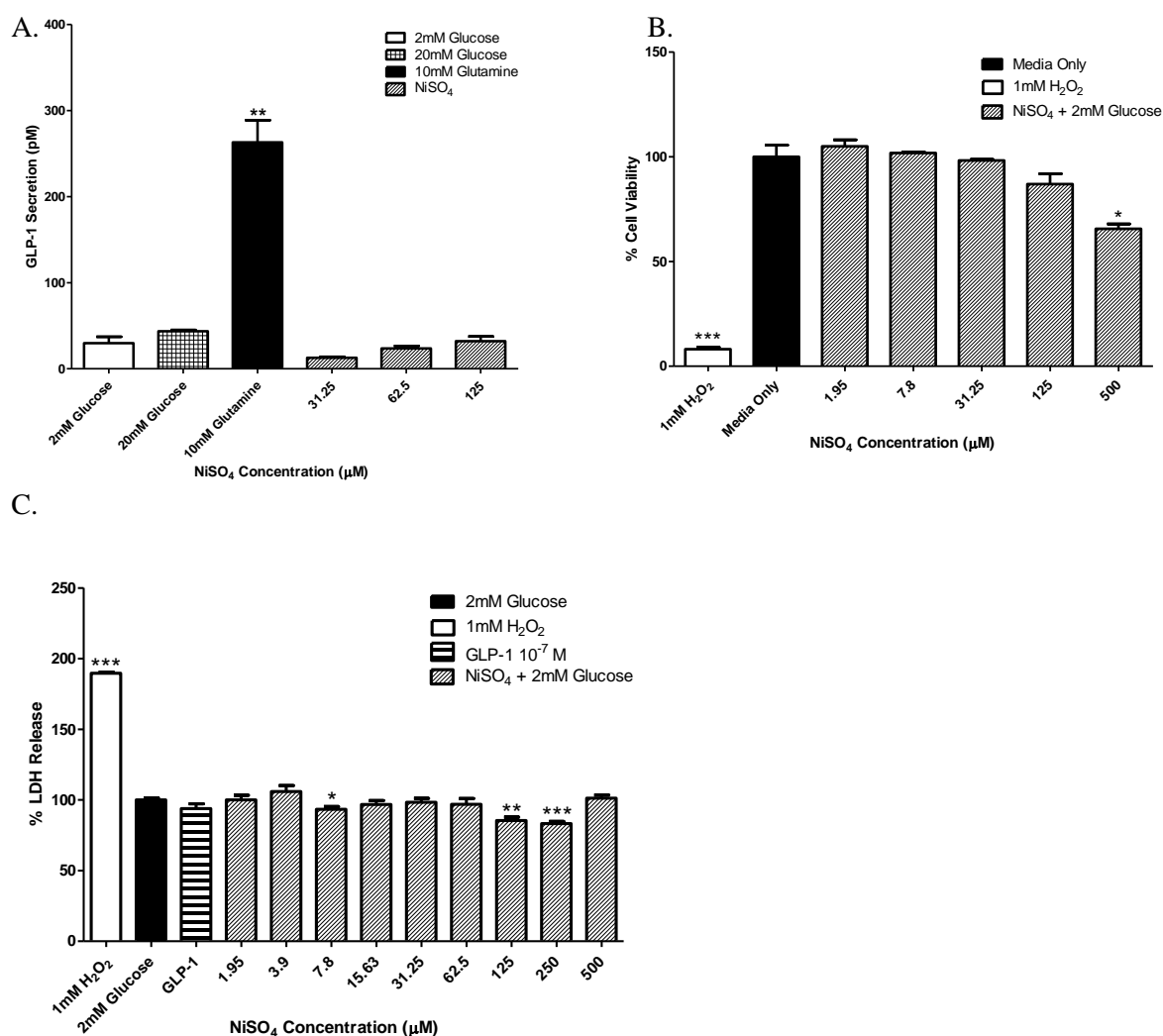


B.



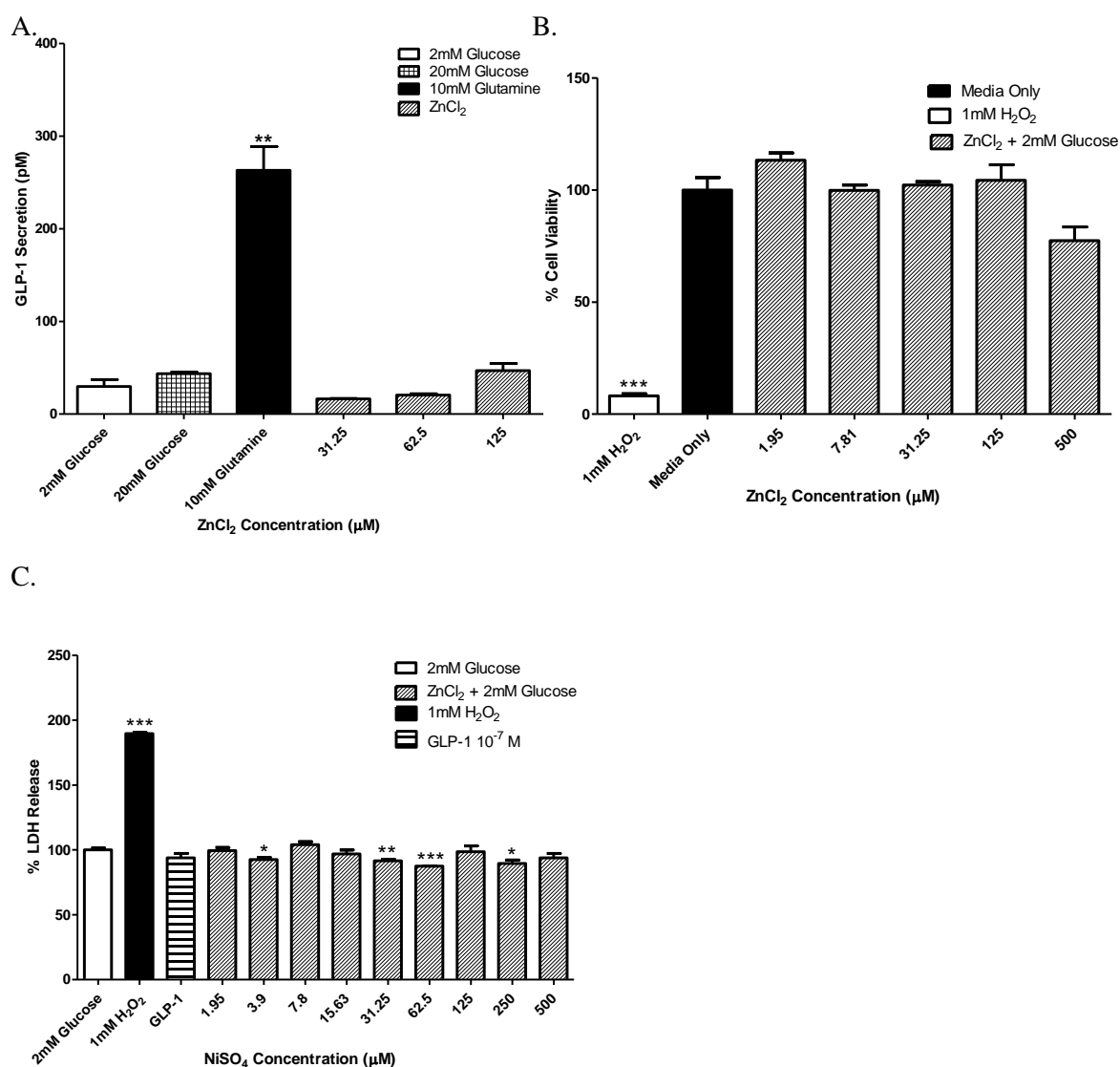
Effect of GPR39 agonist CuCl_2 (1.95-500 μM) on GLP-1 secretion (A) and cell viability (B) in 2mM glucose in GLUTag cells. Cell viability was compared to untreated cells in culture growth medium. GLP-1 secretion was measured using ELISA while cell viability was measured using MTT assay. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability.

Figure 4.2: Effects of GPR39 agonist NiSO_4 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



Effect of GPR39 agonist NiSO_4 (1.95-500 μM) on GLP-1 secretion (A) and cell viability (B and C) in 2mM glucose in GLUTag cells. Cell viability was compared to untreated cells in culture growth medium. GLP-1 secretion was measured using ELISA and cell viability was measured using MTT and (C) LDH assay. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability.

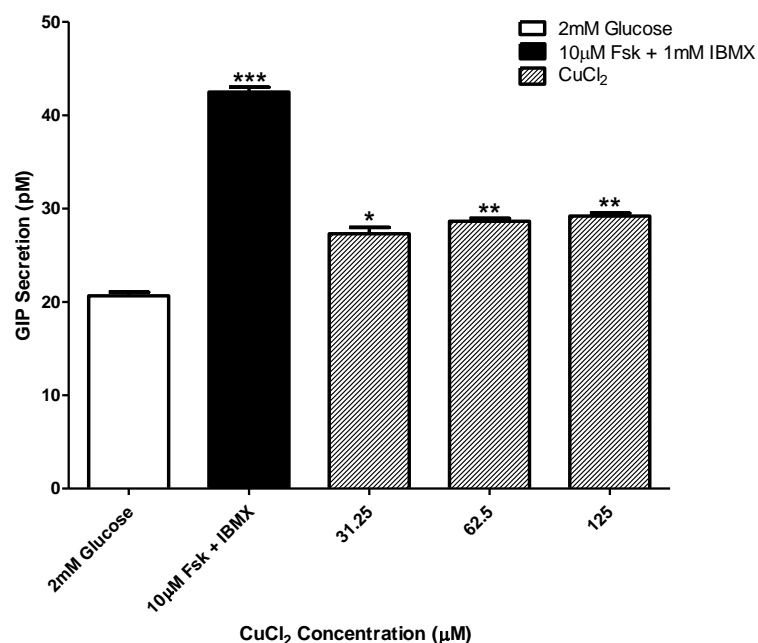
Figure 4.3: Effects of GPR39 agonist ZnCl_2 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



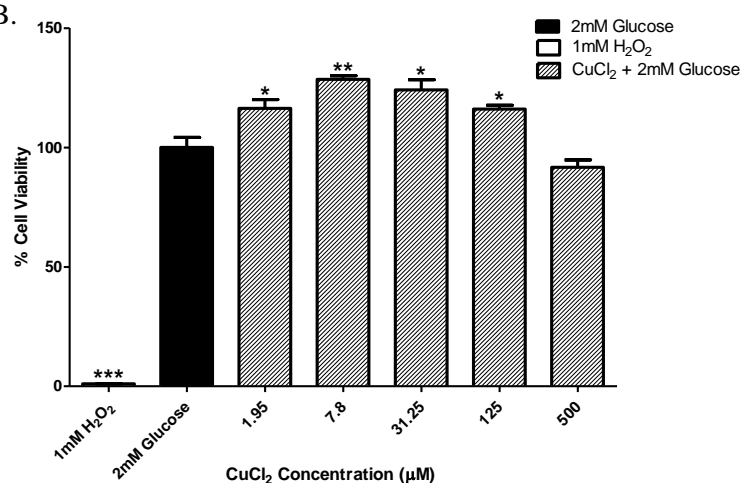
Effect of GPR39 agonist ZnCl_2 (1.95-500 μM) on GLP-1 secretion (A) and cell viability (B and C) at 2mM glucose in GLUTag cells. Cell viability was compared to untreated cells in culture growth medium. GLP-1 secretion was measured using ELISA and cell viability was measured using MTT and LDH assay. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. *p<0.05, **p<0.01 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability.

Figure 4.4: Effects of GPR39 agonist CuCl_2 on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



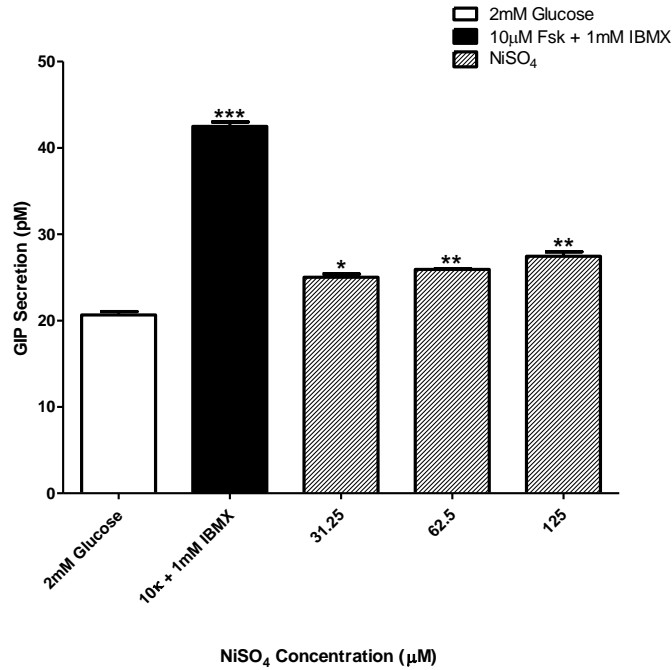
B.



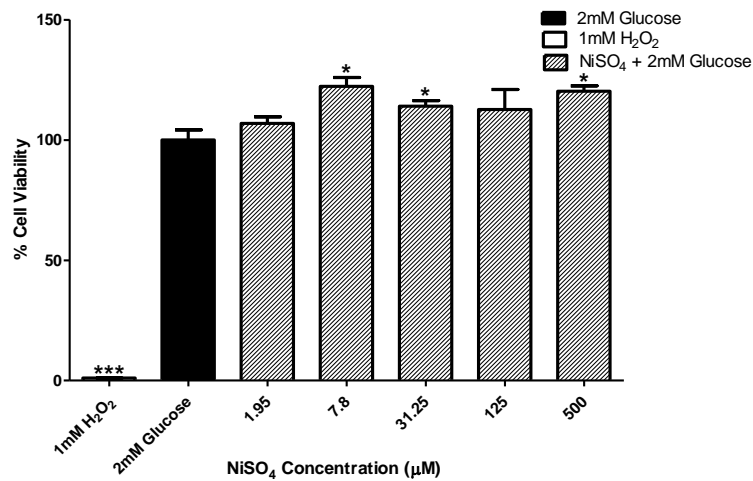
Effect of GPR39 agonist CuCl_2 (1.95-500µM) on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. GIP secretion was measured using ELISA and cell viability was measured using MTT assay. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 4.5: Effects of GPR39 agonist NiSO₄ on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



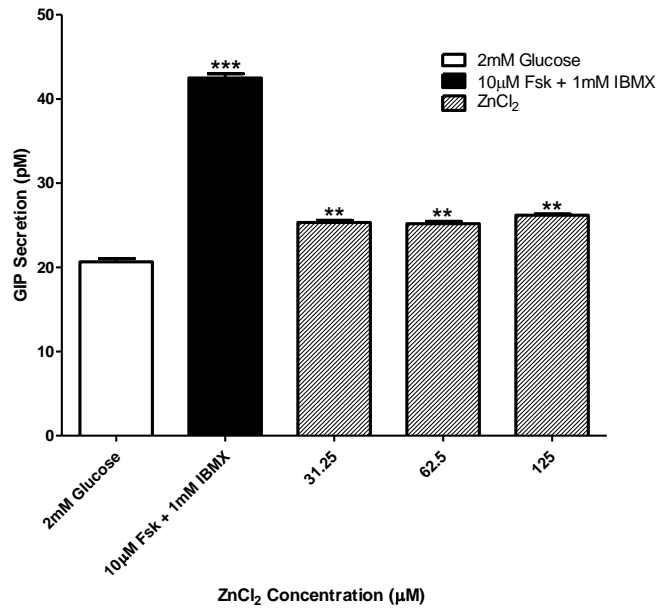
B.



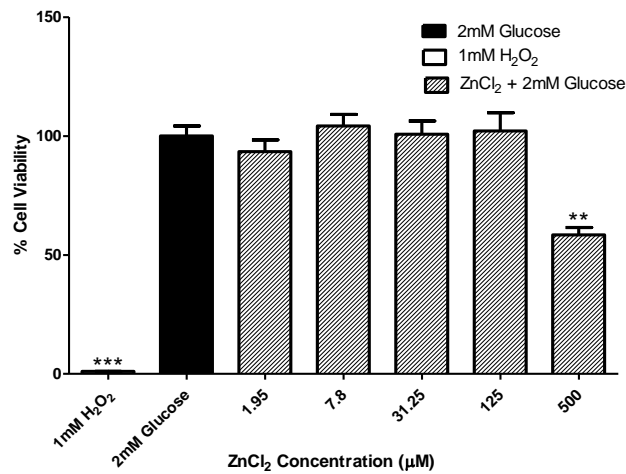
Effect of GPR39 agonist NiSO₄ (1.95-500M) on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. *** p<0.001 compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 4.6: Effects of GPR39 agonist ZnCl_2 on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



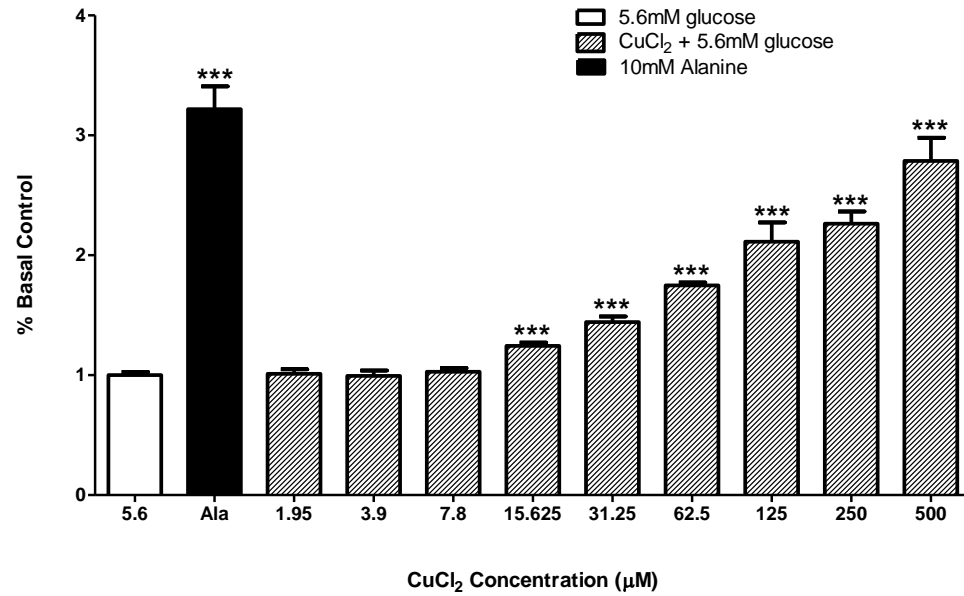
B.



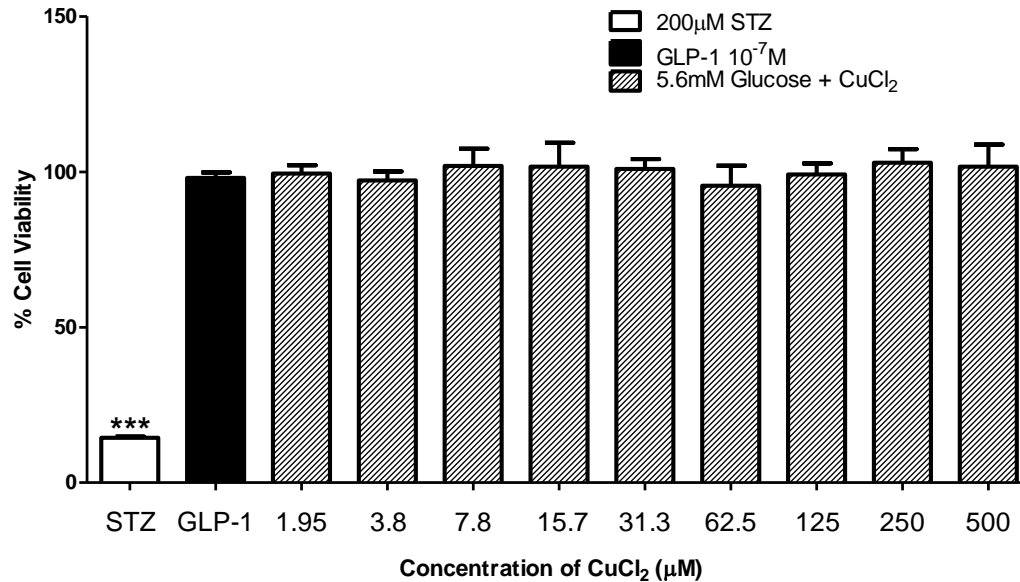
Effect of GPR39 agonist ZnCl_2 (1.95-500µM) on GIP secretion (A) and cell viability (10^{-12} - 10^{-4} M) (B) at 2mM glucose in pGIPneo STC-1 cells. GIP secretion was measured using ELISA and cell viability was measured using MTT assay. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 4.7: Effects of GPR39 agonist CuCl₂ on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



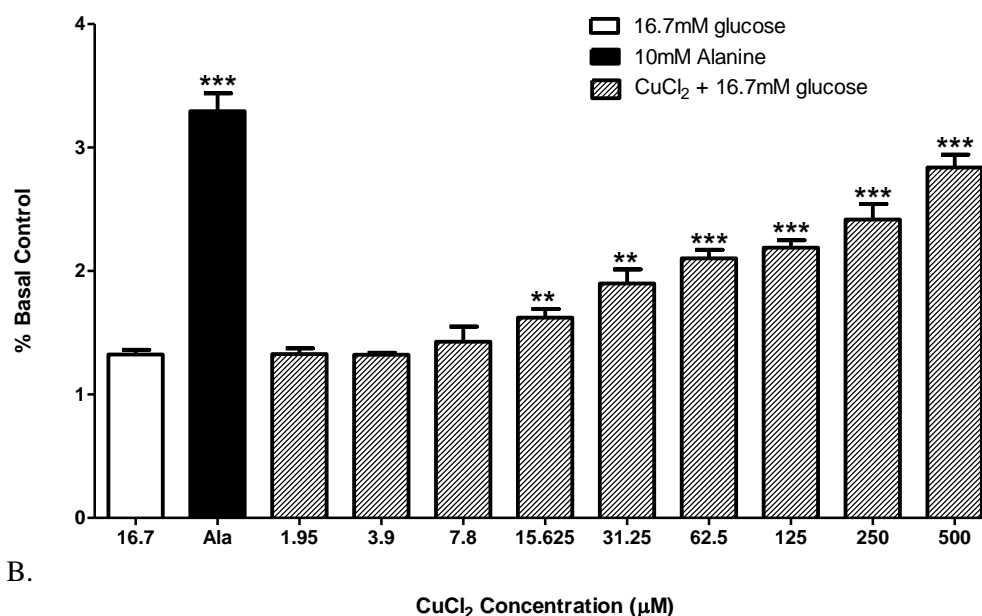
B.



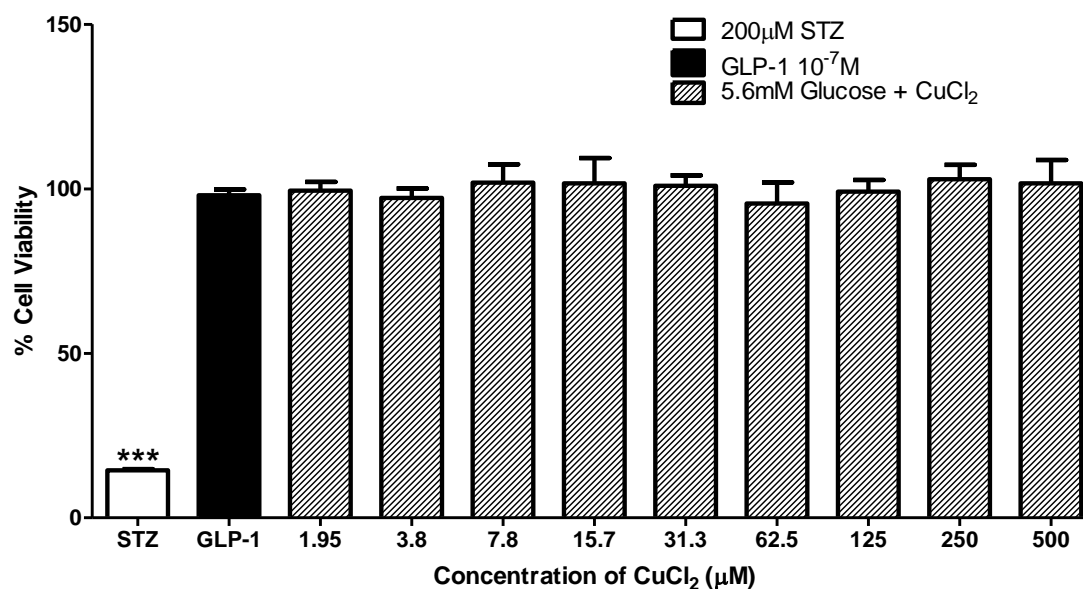
Effect of CuCl₂ (1.95-500μM) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean ± SEM (n=8) for insulin secretion and (n=3) for cell viability. *** p<0.001 compared to basal glucose control for insulin secretion and GLP-1 (10⁻⁷M) non-toxic control for cell viability.

Figure 4.8: Effects of GPR39 agonist CuCl_2 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.

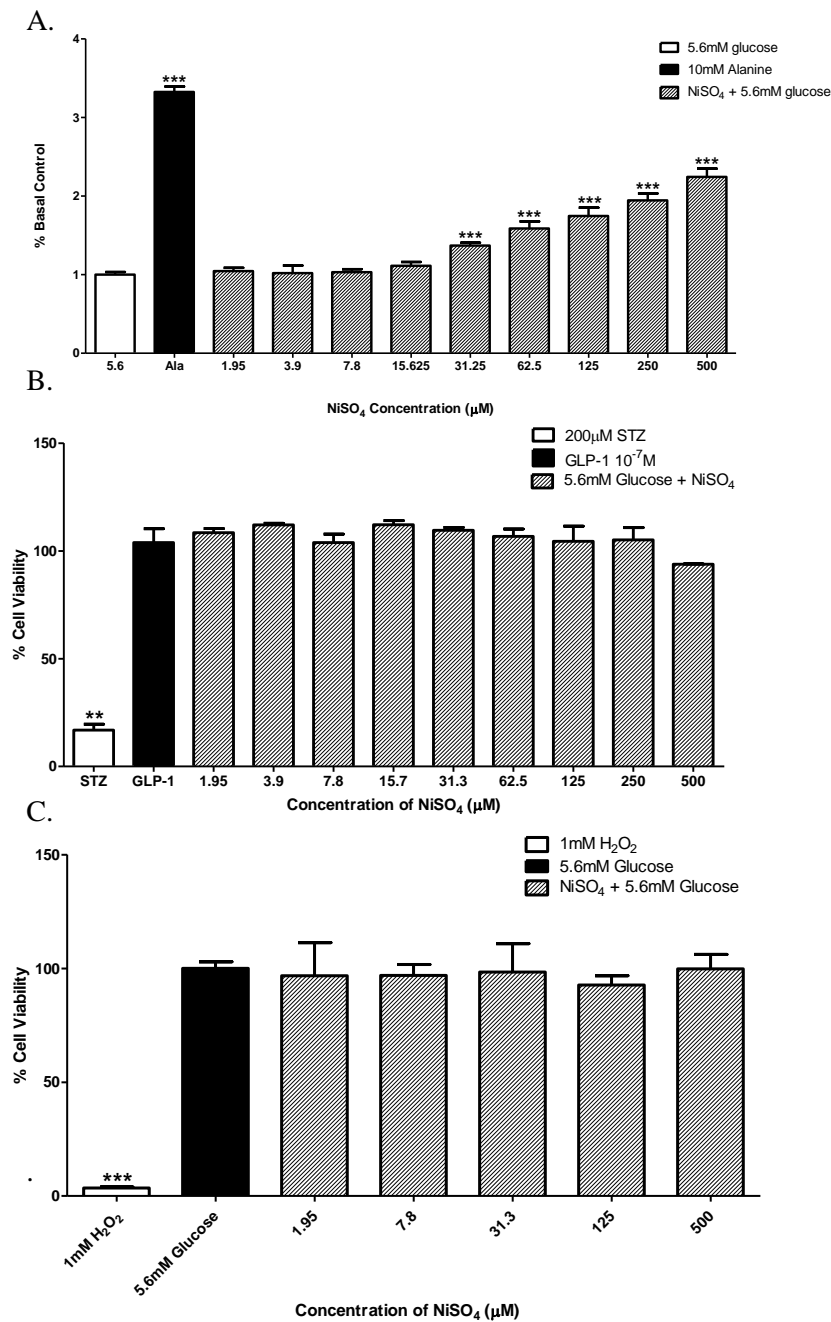


B.



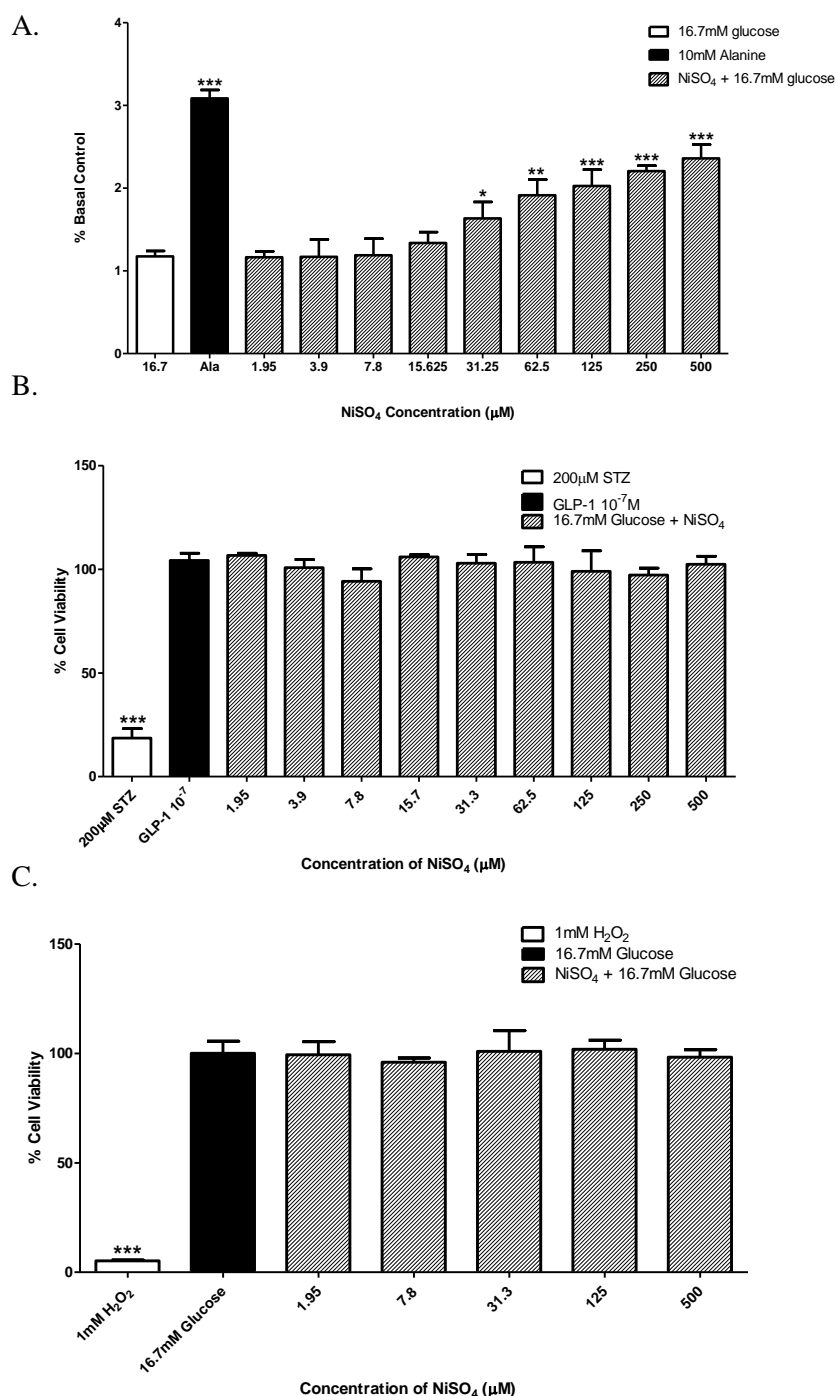
Effect of CuCl_2 (1.95-500 μM) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7}M) non-toxic control for cell viability.

Figure 4.9: Effects of GPR39 agonist NiSO₄ on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose



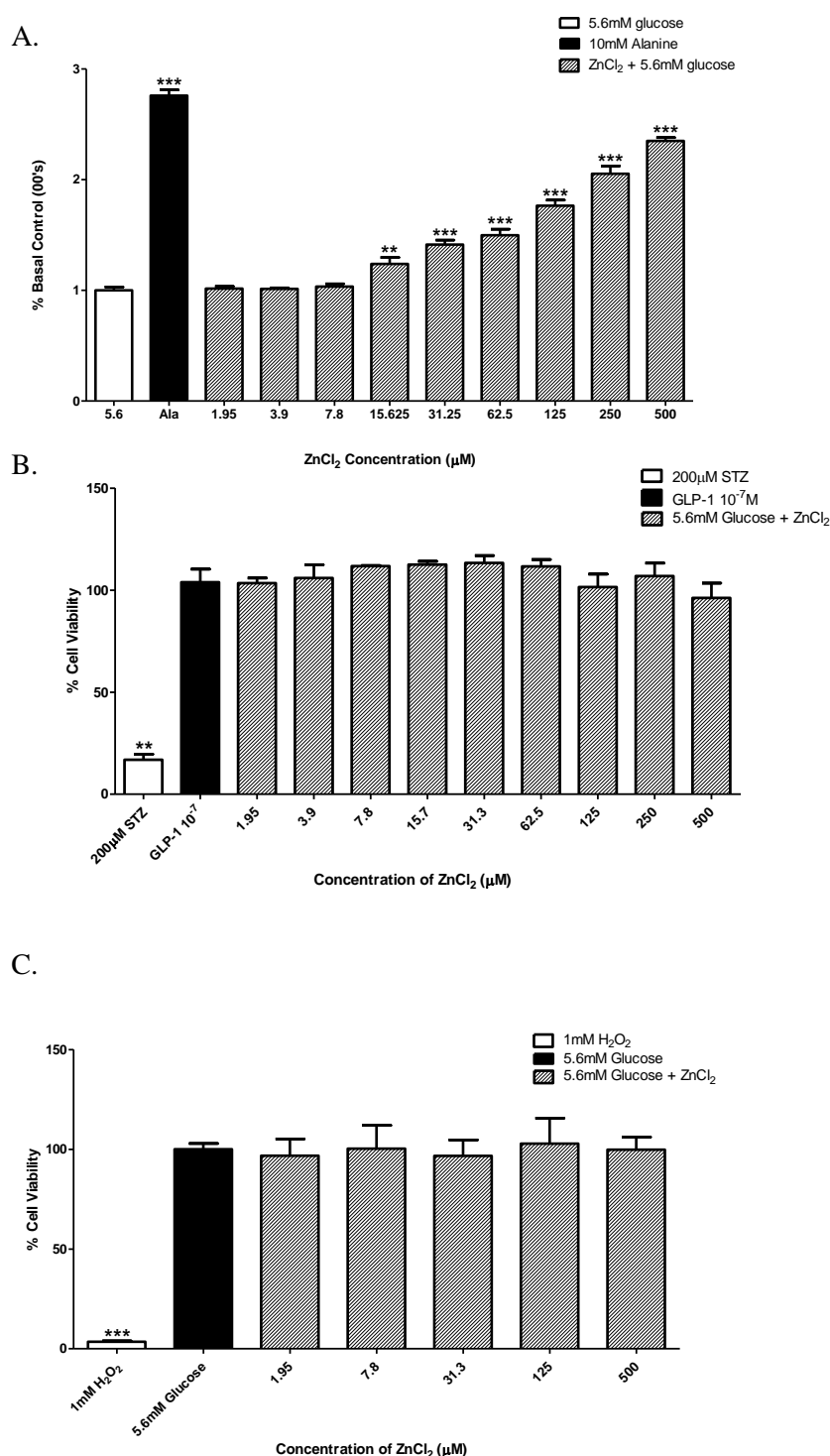
Effect of NiSO₄ (1.95-500μM) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue and MTT Assay. Results are mean ± SEM (n=8) for insulin secretion and (n=3) for cell viability. ** p<0.01 and *** p<0.001 compared to basal glucose control for insulin secretion, GLP-1 (10⁻⁷M) non-toxic control for Alamar Blue assay and 5.6mM glucose vehicle control for MTT assay.

Figure 4.10: Effects of GPR39 agonist NiSO_4 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



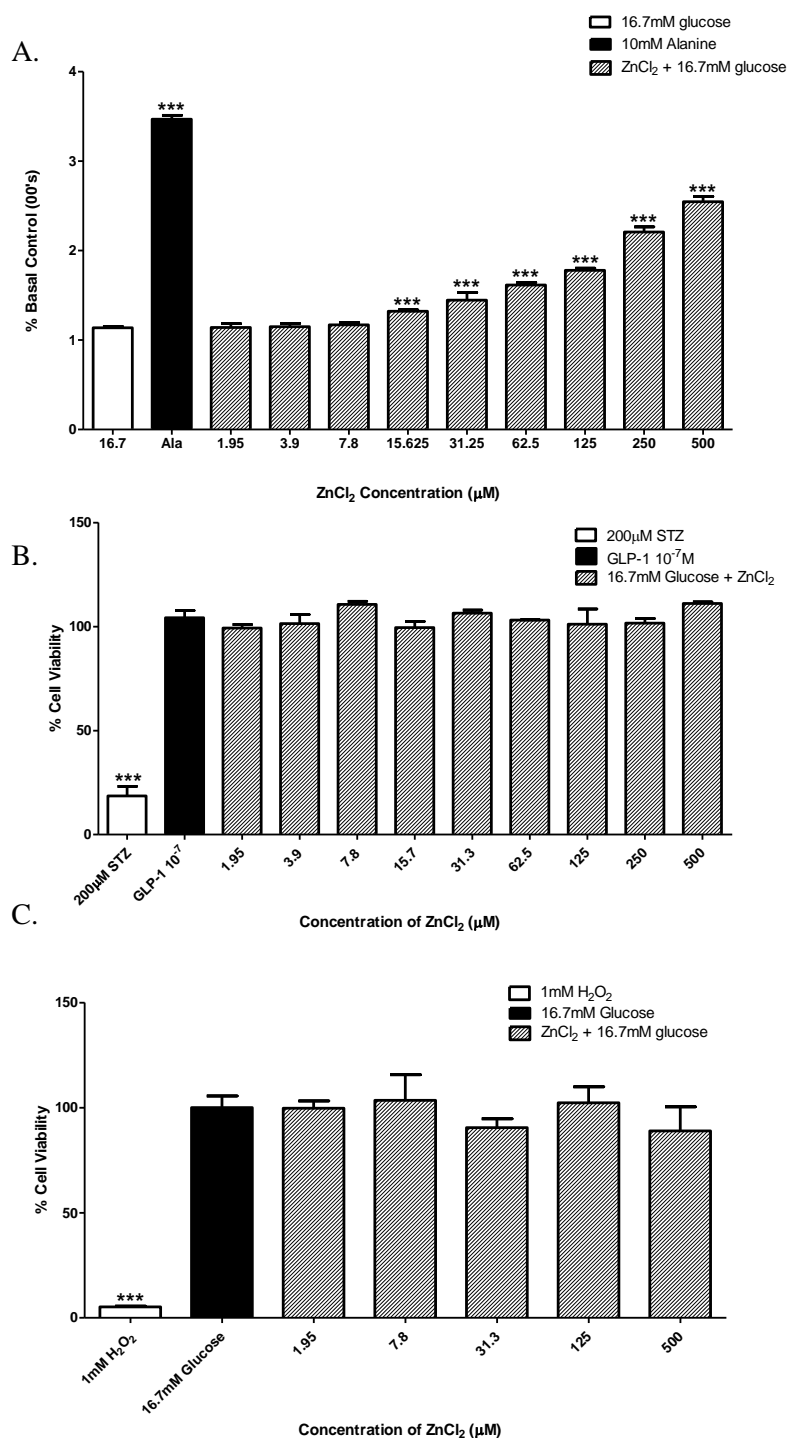
Effect of ZnCl_2 (1.95-500 μM) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue and MTT Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion, GLP-1 (10^{-7}M) non-toxic control for Alamar Blue assay and 16.7m glucose vehicle control for MTT assay.

Figure 4.11: Effects of GPR39 agonist ZnCl_2 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose



Effect of ZnCl_2 (1.95-500 μM) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue and MTT Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion, GLP-1 (10^{-7}M) non-toxic control for Alamar Blue assay and 5.6mM glucose vehicle control for MTT assay.

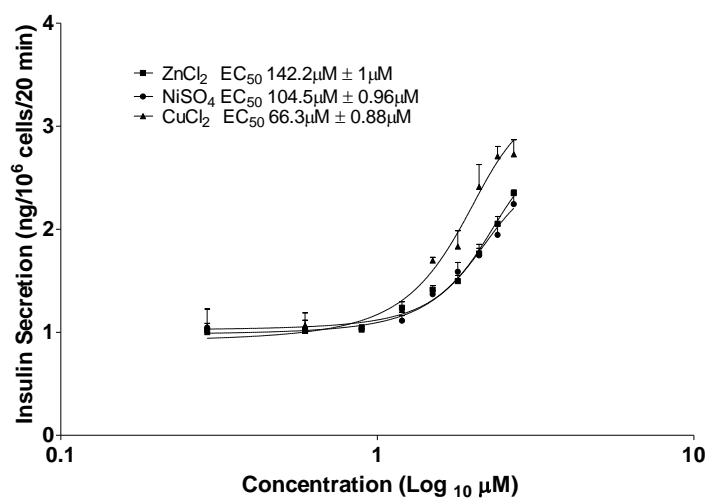
Figure 4.12: Effects of GPR39 agonist ZnCl_2 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



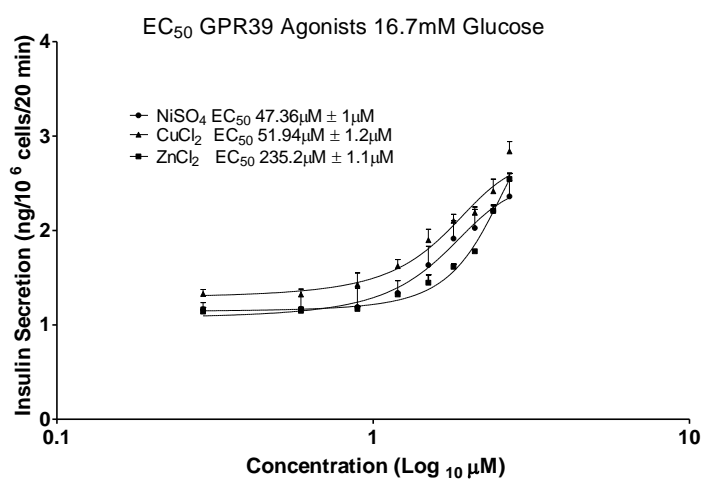
Effect of ZnCl_2 (1.95-500μM) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue and MTT Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. *** p<0.001 compared to basal glucose control for insulin secretion, GLP-1 (10^{-7} M) non-toxic control for Alamar Blue assay and 16.7mM glucose vehicle control for MTT assay.

Figure 4.13: EC₅₀ of GPR39 agonists CuCl₂, NiSO₄ and ZnCl₂ in BRIN-BD11 cells

A.

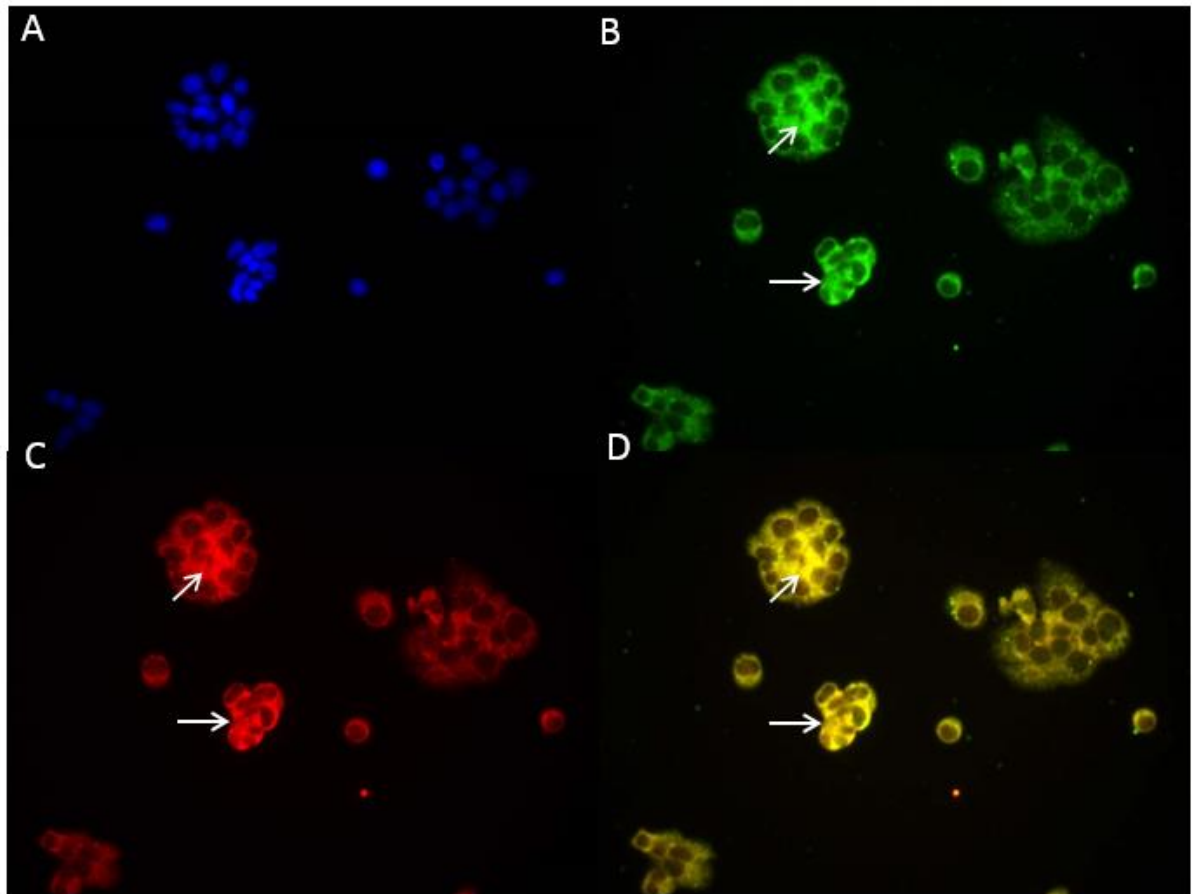


B.



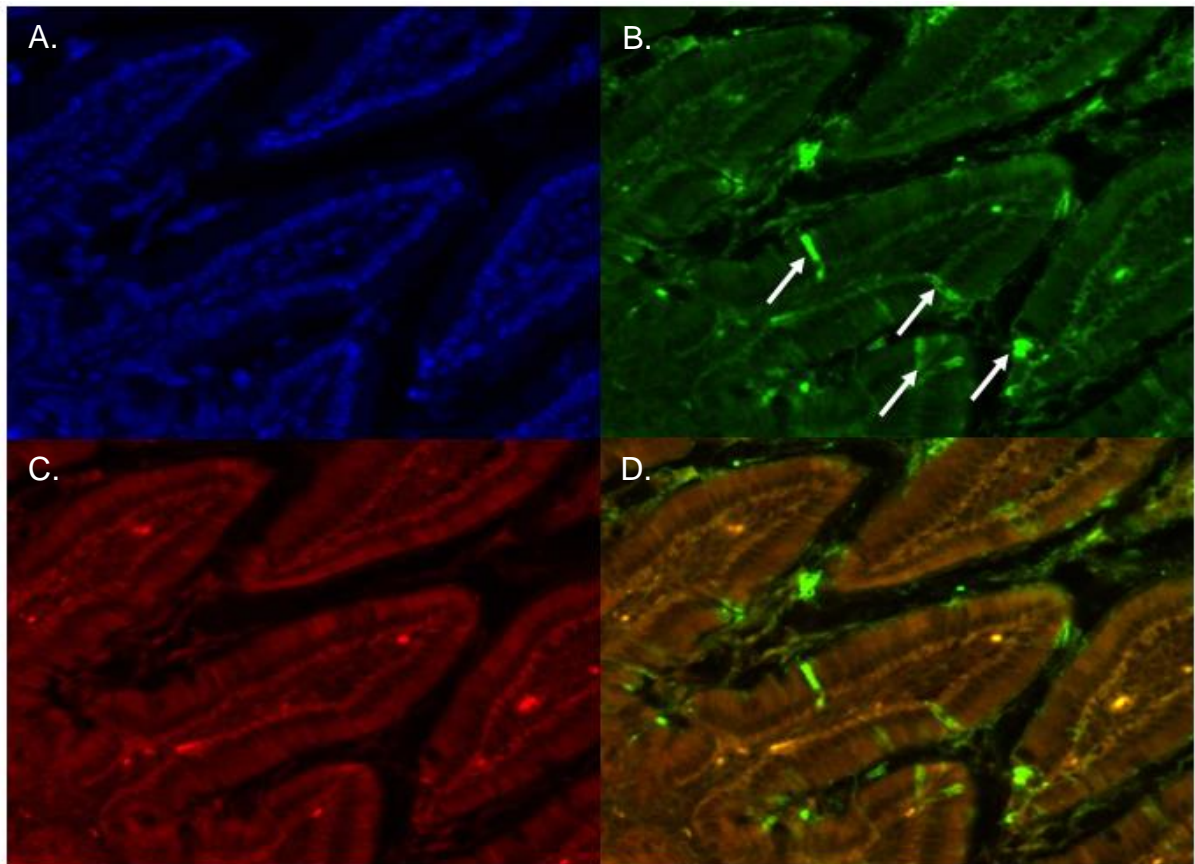
EC₅₀ values for GPR39 agonists CuCl₂, NiSO₄ and ZnCl₂ in BRIN-BD11 cells in 5.6mM glucose (A) and 16.7mM glucose (B).

Figure 4.14: Double immunofluorescence staining of GLP-1 and GPR39 in intestinal GLUTag cells



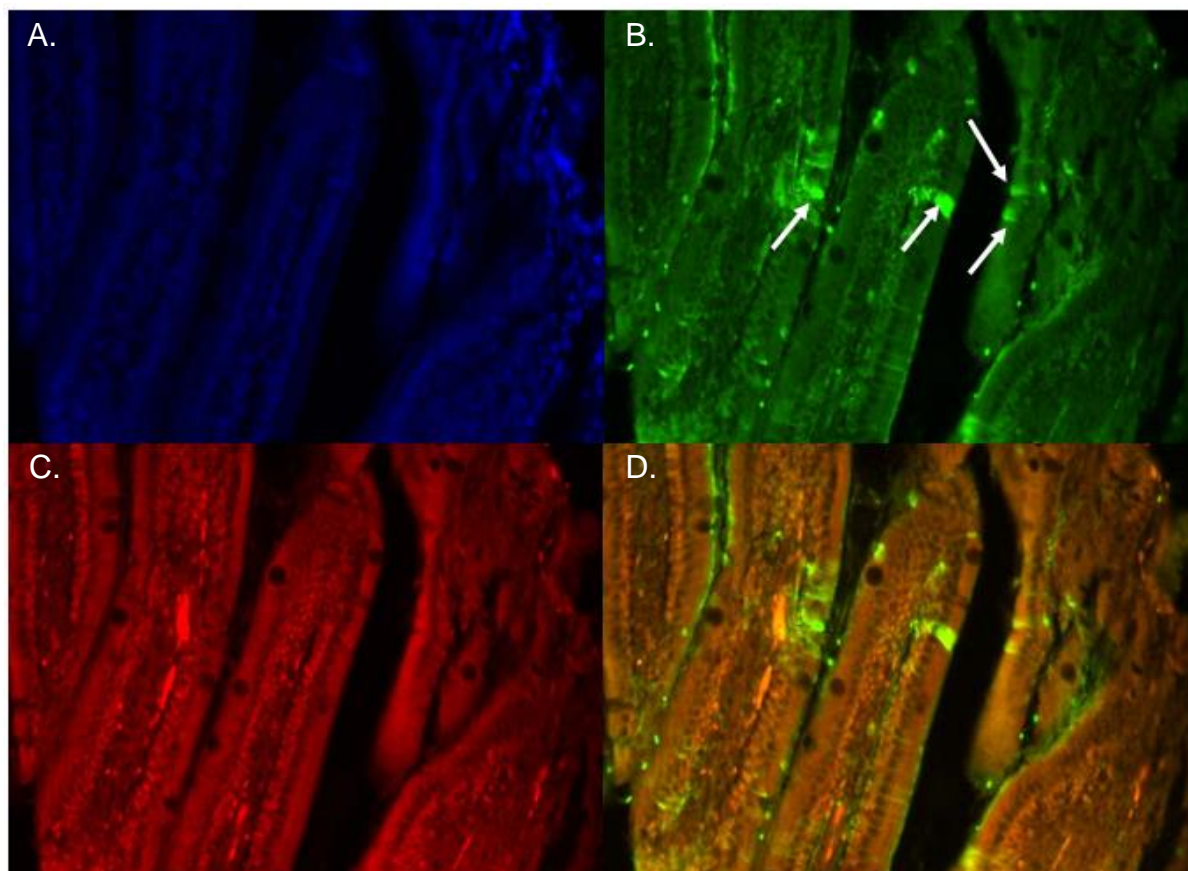
Distribution of (A) DAPI, (B) GLP-1, (C) GPR39 and (D) combined fluorescence of GLP-1 and GPR39 in GLUTag cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.15: Double immunofluorescence staining of GLP-1 and GPR39 in lean NIH Swiss mouse small intestine



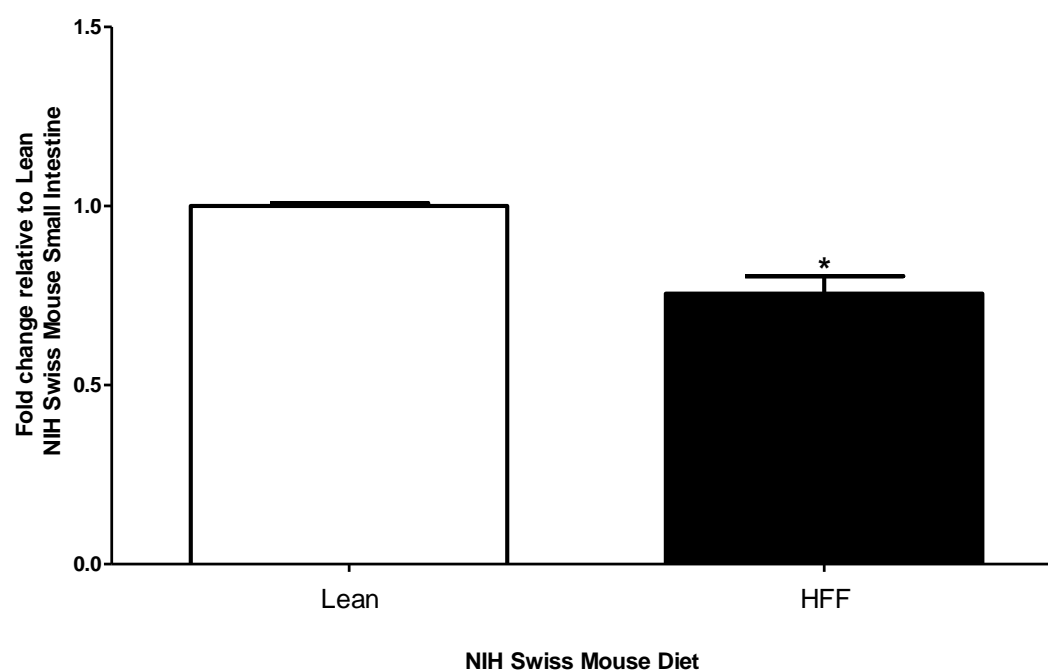
Distribution of (A) DAPI, (B) GLP-1, (C) GPR39 and (D) combined fluorescence of GLP-1 and GPR39 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.16: Double immunofluorescence staining of GLP-1 and GPR39 in HFF NIH Swiss mouse small intestine



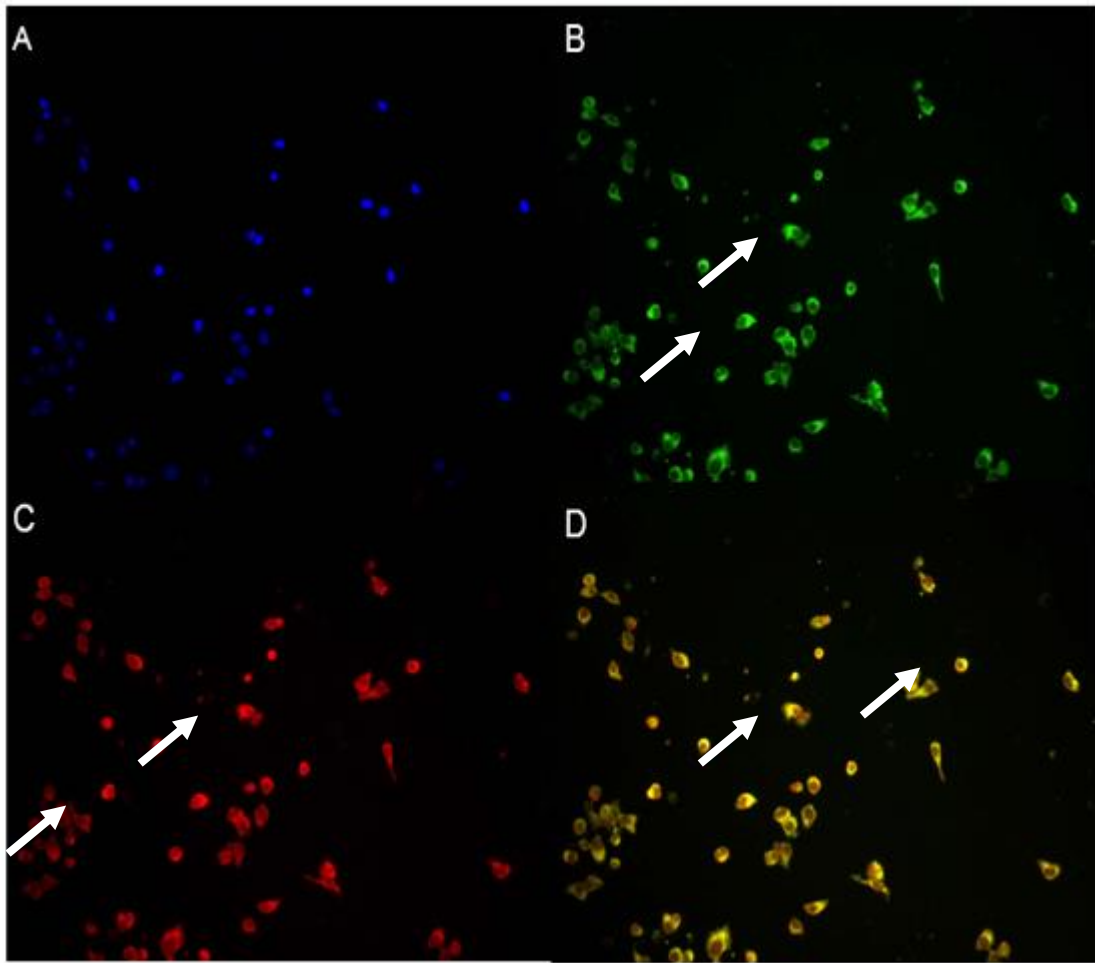
Distribution of (A) DAPI, (B) GLP-1, (C) GPR39 and (D) combined fluorescence of GLP-1 and GPR39 in HFF NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.17: GPR39 gene expression in lean and HFF NIH Swiss mouse small intestine



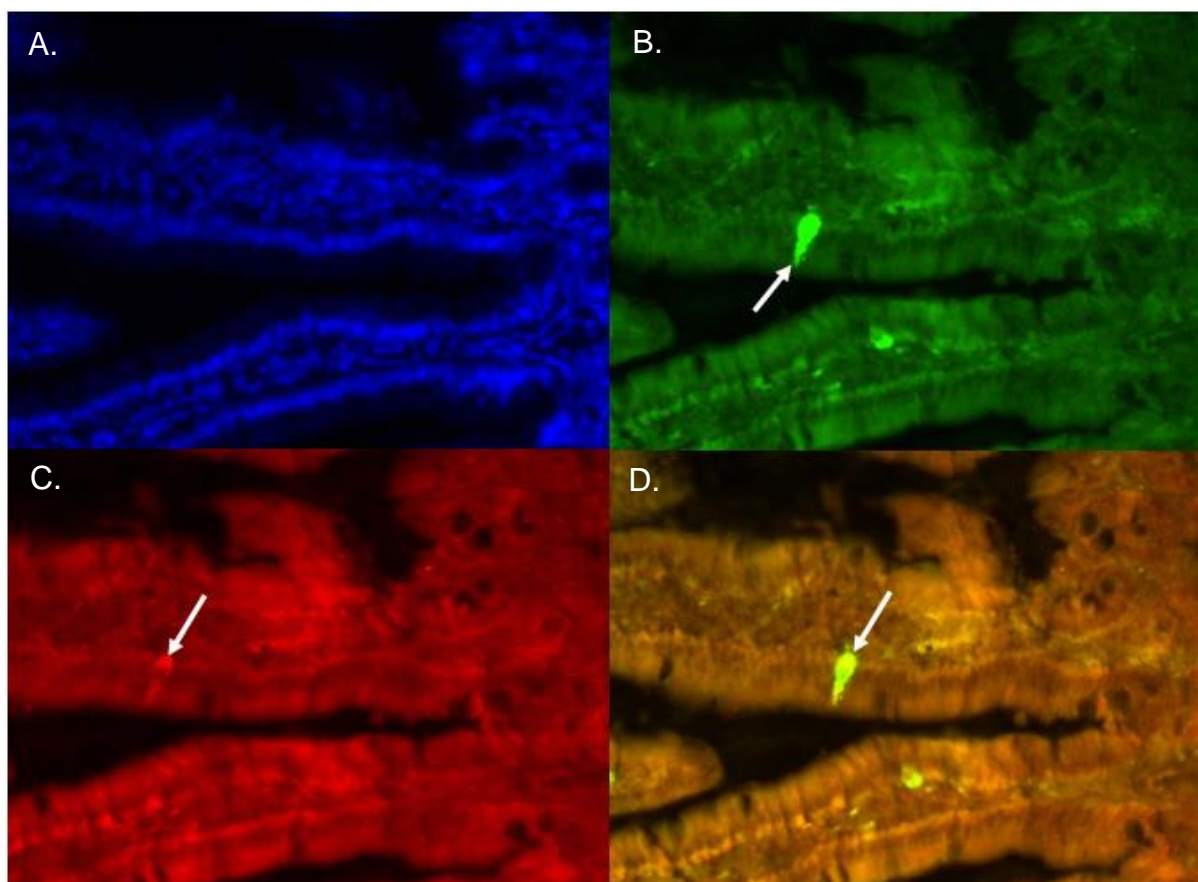
Effect of HFF diet on GPR39 gene expression in male NIH Swiss mouse small intestine in NIH Swiss mice (n=6). * $p < 0.05$.

Figure 4.18: Double immunofluorescence staining of GIP and GPR39 in pGIPneo STC-1 cells



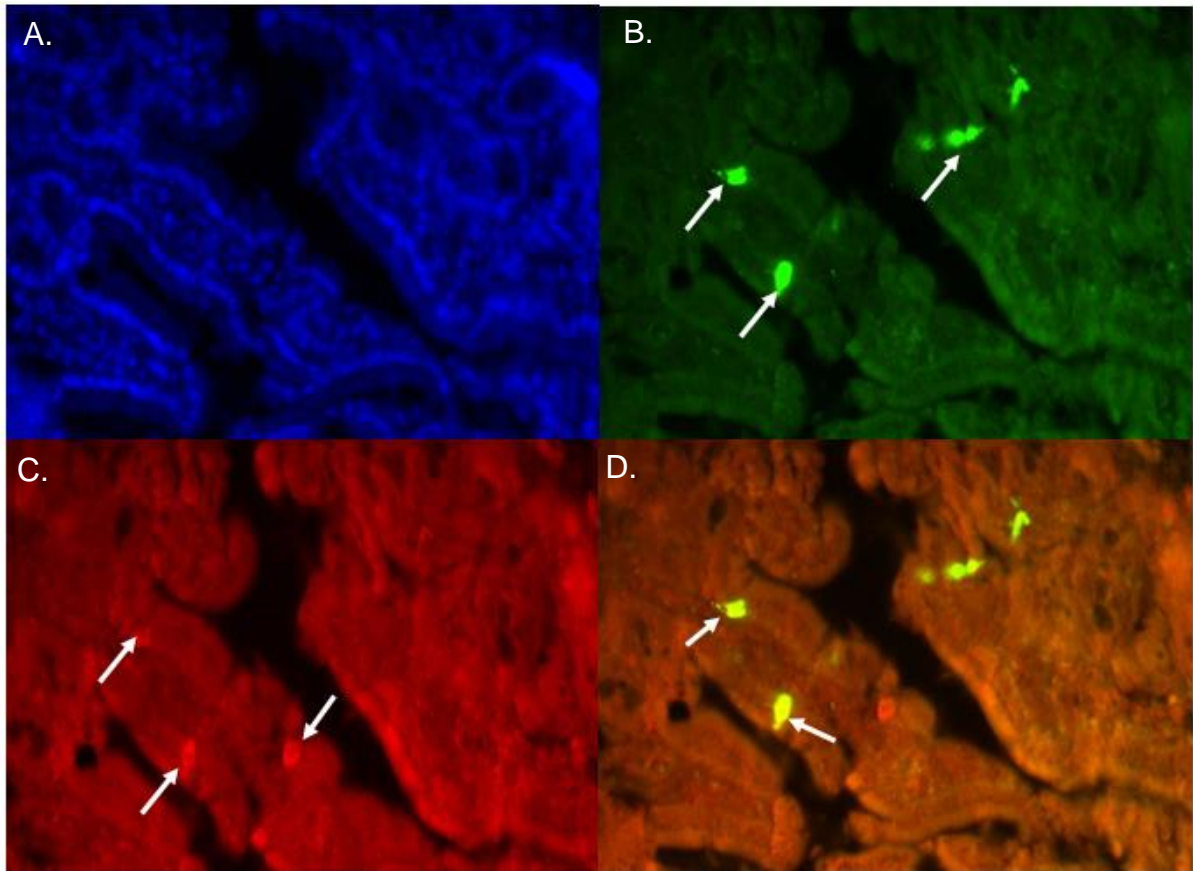
Distribution of (A) DAPI, (B) GIP, (C) GPR39 and (D) combined fluorescence of GIP and GPR39 in pGIPneo STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.19: Double immunofluorescence staining of GIP and GPR39 in lean NIH Swiss mouse small intestine



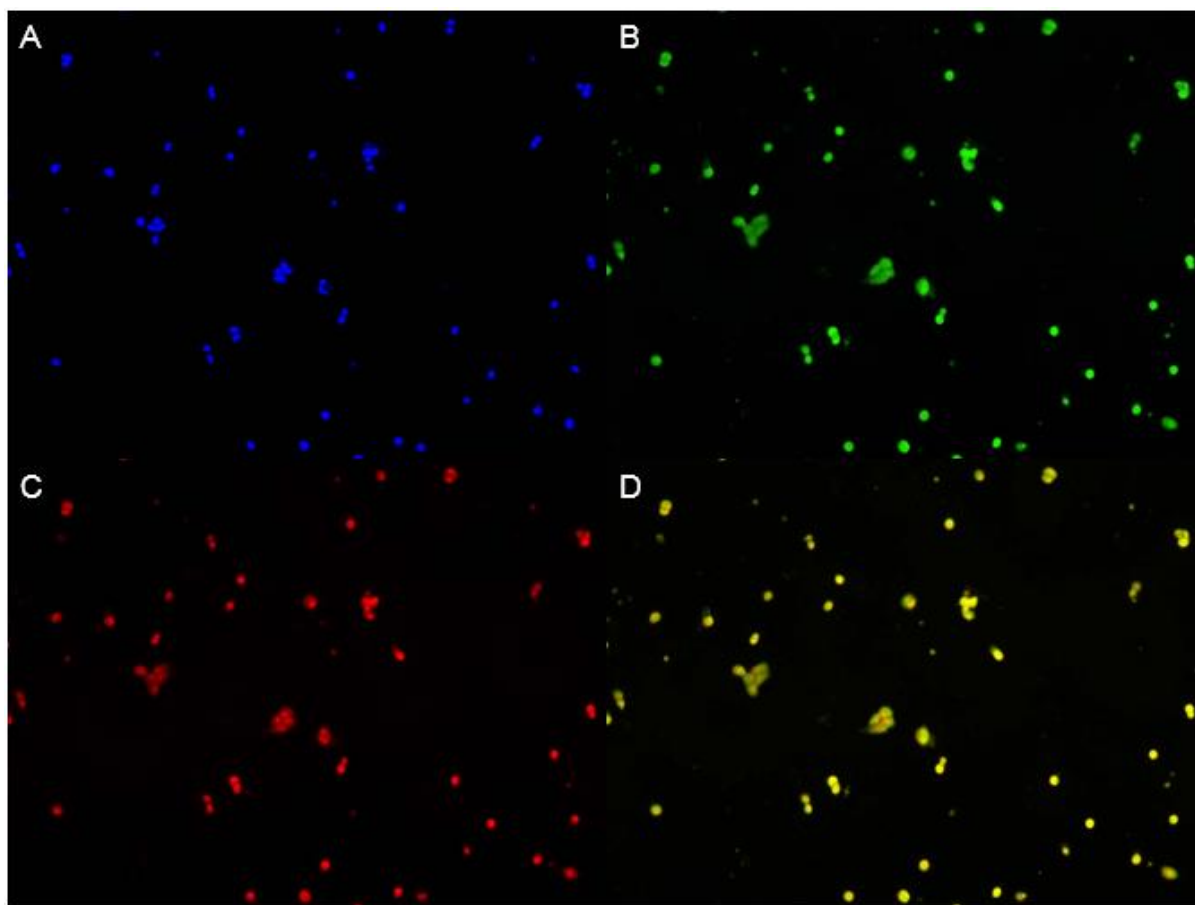
Distribution of (A) DAPI, (B) GIP, (C) GPR39 and (D) combined fluorescence of GIP and GPR39 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.20: Double immunofluorescence staining of GIP and GPR39 in HFF NIH Swiss mouse small intestine



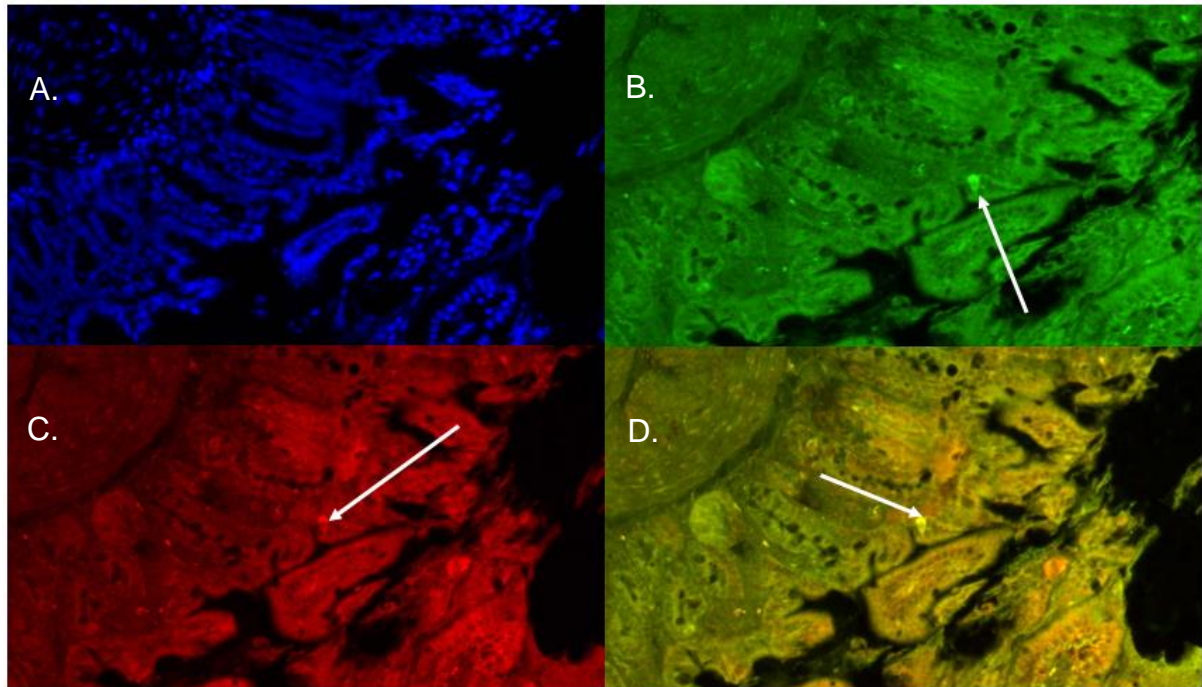
Distribution of (A) DAPI, (B) GIP, (C) GPR39 and (D) combined fluorescence of GIP and GPR39 in HFF NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.21: Double immunofluorescence staining of PYY and GPR39 in STC-1 cells



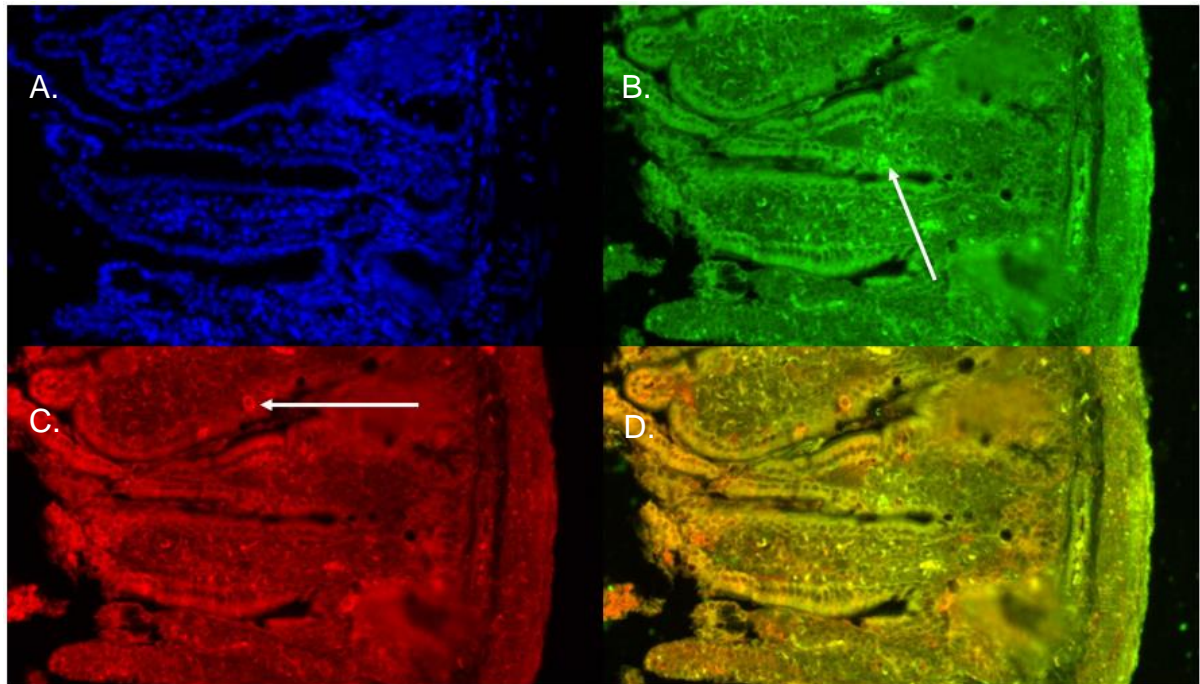
Distribution of (A) DAPI, (B) PYY, (C) GPR39 and (D) combined fluorescence of PYY and GPR39 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.22: Double immunofluorescence staining of PYY and GPR39 in lean NIH Swiss mouse small intestine



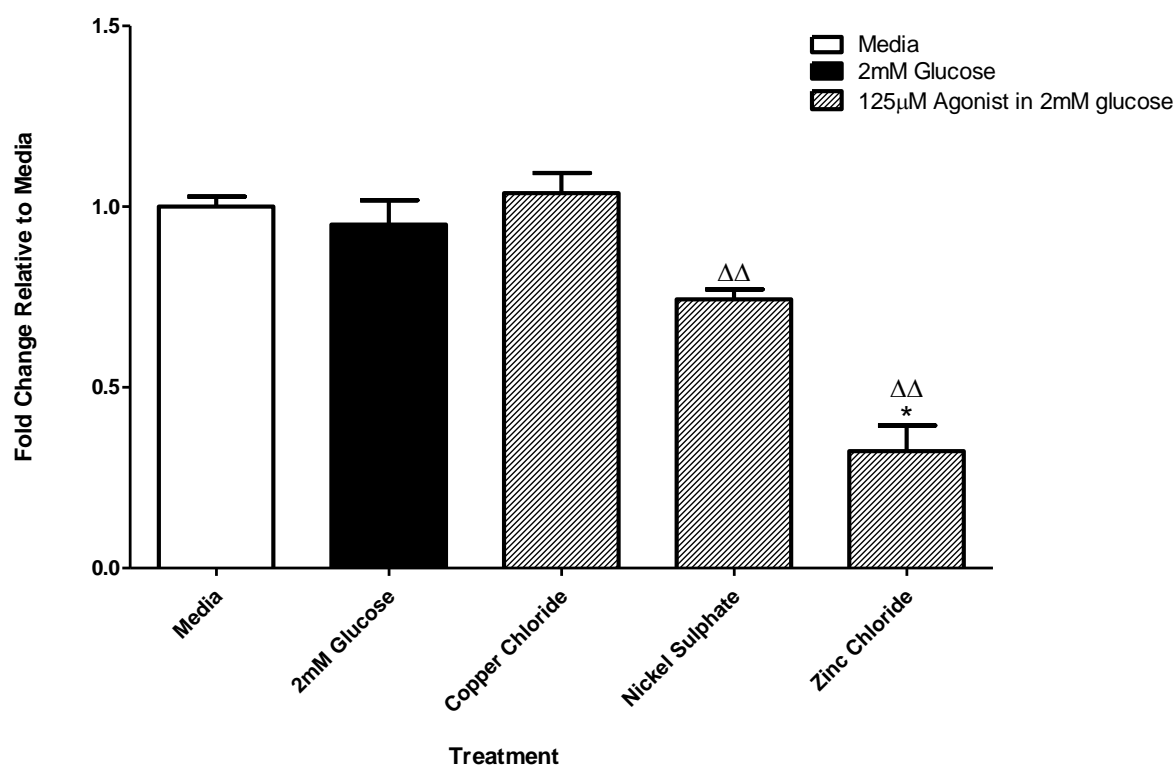
Distribution of (A) DAPI, (B) PYY, (C) GPR39 and (D) combined fluorescence of PYY and GPR39 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.23: Double immunofluorescence staining of PYY and GPR39 in HFF NIH Swiss mouse small intestine



Distribution of (A) DAPI, (B) PYY, (C) GPR39 and (D) combined fluorescence of PYY and GPR39 in HFF NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 4.24: Effect of GPR39 agonists on proglucagon gene expression in GLUTag cells

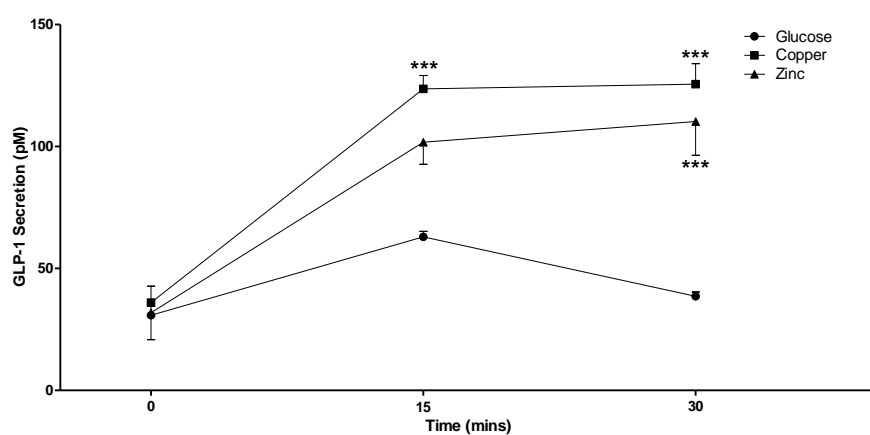


Effects of GPR39 agonists on GPR39 gene expression in intestinal GLUTag cells at 2mM glucose.

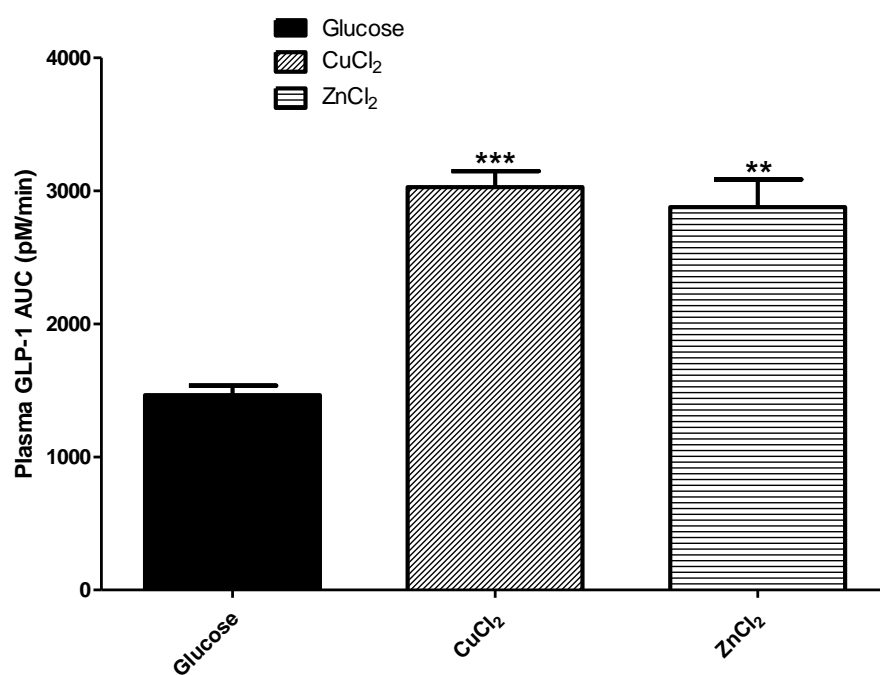
* $p < 0.01$ compared to vehicle control. $\Delta\Delta$ $p < 0.05$ compared to untreated control.

Figure 4.25: Effects of GPR39 agonists on plasma GLP-1 in male Swiss TO mice

A.



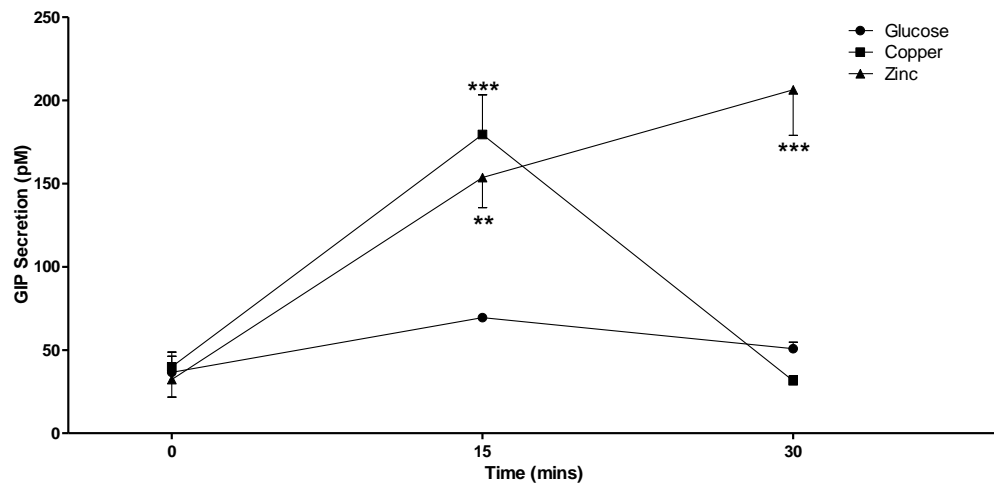
B.



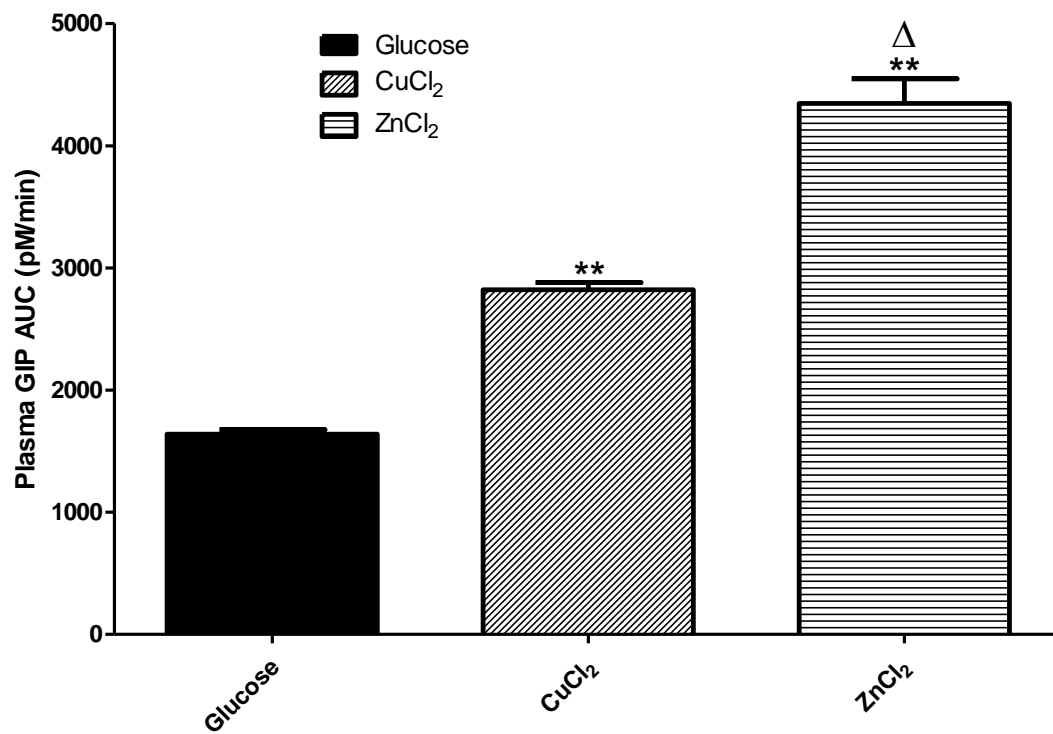
Glucose (18mmol/kg BW) or glucose in combination with a GPR39 agonist (0.1μmol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GLP-1 of mice treated with CuCl₂ and ZnCl₂ (B) AUC of A. ** p<0.01 and *** p<0.001 compared to glucose administration.

Figure 4.26: Effects of GPR39 agonists on plasma GIP in male Swiss TO mice

A.

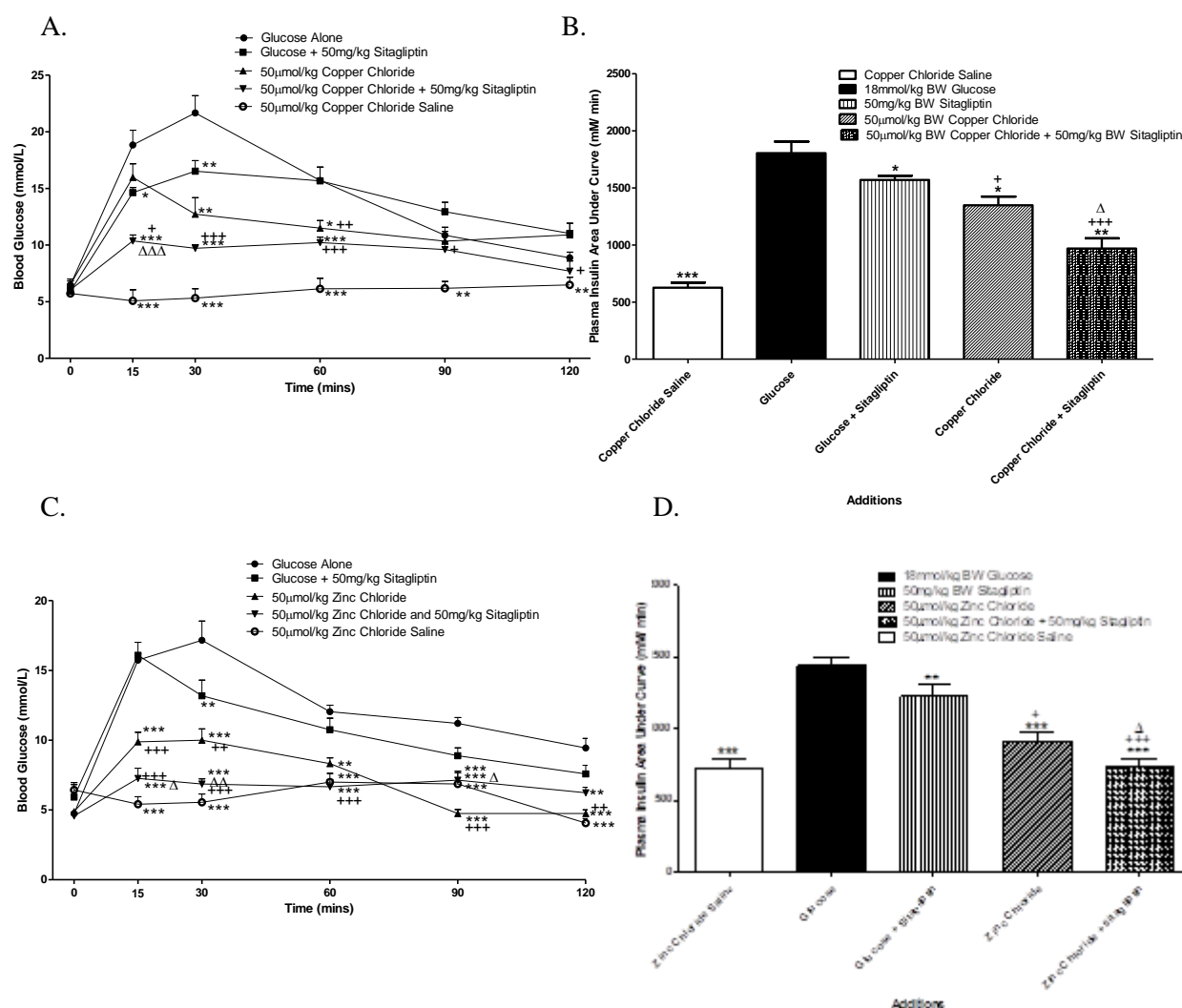


B.



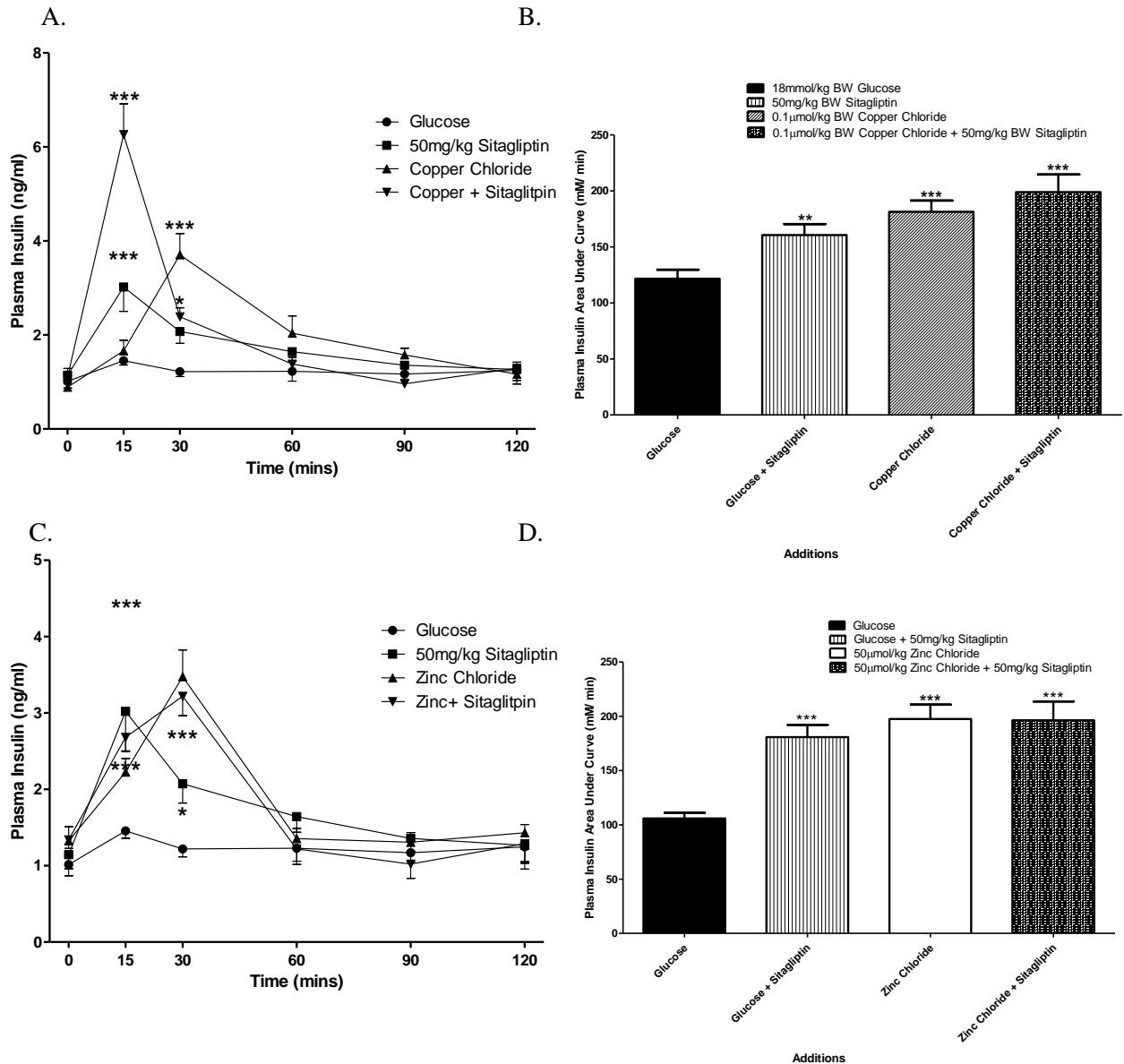
Glucose (18mmol/kg BW) or glucose in combination with a GPR39 agonist (0.1μmol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GIP of mice treated with CuCl₂ and ZnCl₂ (B) AUC of A. ** p<0.01 and *** p<0.001 compared to glucose. Δ p<0.05 compared to CuCl₂

Figure 4.27: Acute effects of oral administration of GPR39 agonists on blood glucose in male Swiss TO mice



Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with a GPR39 agonist (50μmol/kg BW), glucose in combination with a GPR39 agonist and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with a GPR39 agonist were all administered orally to male Swiss TO mice (n=6). (A) Blood glucose of mice treated with CuCl₂ (B) AUC of A (C) Blood glucose of mice treated with ZnCl₂ (D) AUC of C. * p<0.05, ** p<0.01 and *** p<0.001 compared to glucose treatment alone. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to agonist treatment alone. + p<0.05, ++ p<0.01 and +++ p<0.001 compared to Sitagliptin treatment alone.

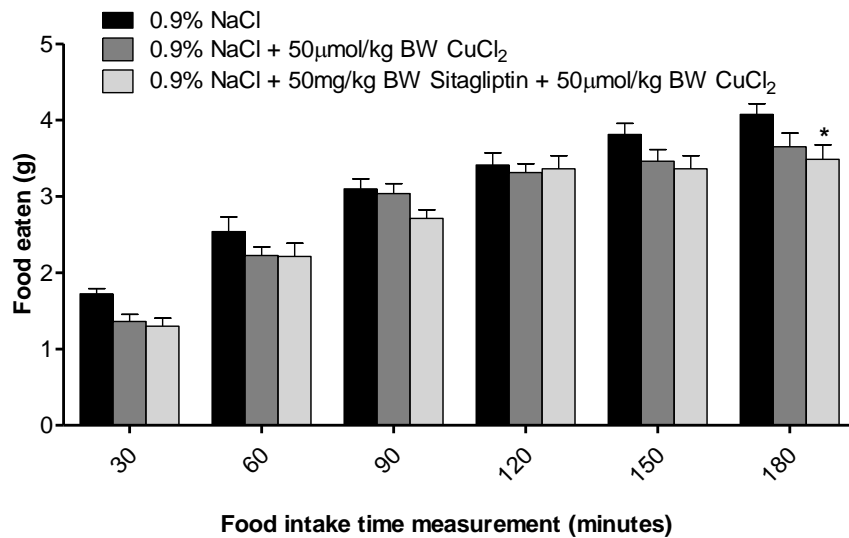
Figure 4.28: Effects of oral administration of GPR39 agonists and GPR39 agonists in combination with Sitagliptin on plasma insulin in male Swiss TO mice



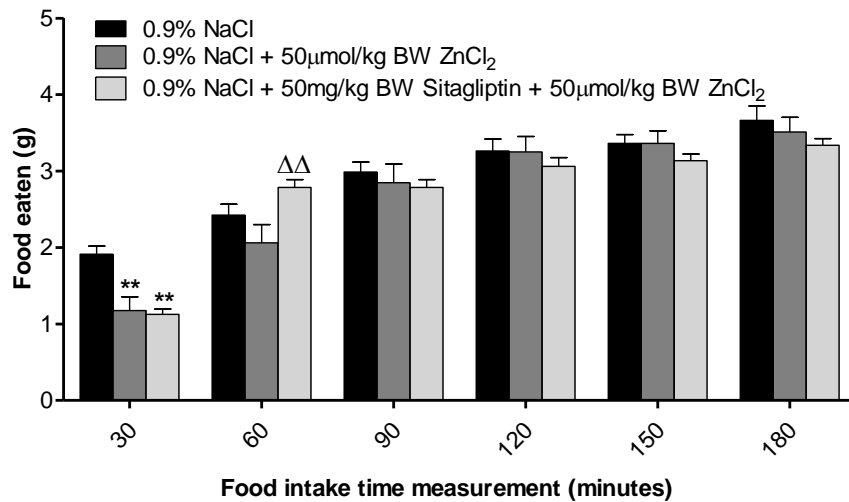
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with a GPR39 agonist (50μmol/kg BW) or glucose in combination with a GPR39 agonist and Sitagliptin Phosphate were administered orally to fasted Swiss TO mice (n=6). (A) Plasma insulin of mice treated with CuCl₂ (B) AUC of A (C) Plasma insulin of mice treated with ZnCl₂ (D) AUC of C. * p<0.05, ** p<0.01 and *** p<0.001 compared to glucose treatment alone. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to agonist treatment alone. + p<0.05, ++ p<0.01 and +++ p<0.001 compared to Sitagliptin treatment alone.

Figure 4.29: Effects of GPR39 agonists on feeding in trained male Swiss TO mice

A.



B.



Saline (0.9%) or saline and a GPR39 agonist (50μmol/kg BW) were administered orally to Swiss TO mice which had been trained to eat for 3 hrs daily (n=8). * p<0.05 and **p<0.01 compared to saline. ΔΔ p<0.01 compared to agonist treatment alone.

Chapter 5

Investigating the effects of GPR120
activation by fatty acids on insulin and
incretin hormone secretion *in vitro* and *in*
vivo

5.1: Overview of results

GPR120 is a GPCR that is expressed throughout the body in organs such as the lungs and brain as well as the gastrointestinal tract and was first found to be expressed in the stomach. There had been some contention over the presence of GPR120 in the pancreas, however recent studies have identified GPR120 as having a role in insulin, glucagon and somatostatin secretion. The current study investigates the role of GPR120 in intestinal hormone secretion from a range of cell lines as well as its role in insulin secretion from BRIN-BD11 cells. The effects of treating GLUTag, pGIPneo STC-1 and BRIN-BD11 cell lines with the GPR120 agonists ALA, DHA and GW9508 at concentrations between 10^{-12} and 10^{-4} M was examined. Cytotoxicity was assessed in GLUTag and pGIPneo STC-1 cells using an MTT assay while in BRIN-BD11 cells this was measured using the Alamar blue assay. Expression of GPR120, GIP, GLP-1 and PYY were determined using double immunohistochemistry of intestinal cells *in vivo* and *in vitro* and confirmed using qPCR. The metabolic *in vivo* effects of agonists DHA and GW9508 were determined at $0.1\mu\text{mol/kg BW}$ both alone and in combination with Sitagliptin (50mg/kg BW) in male Swiss TO mice ($n=6$).

The omega 3 fatty acid DHA increased GLP-1 secretion 113% ($p<0.001$) at 10^{-6} M compared to basal glucose concentrations in GLUTag cells. ALA increased GLP-1 secretion 75.2% ($p<0.01$) at a concentration of 10^{-6} M while GW9508 increased GLP-1 secretion 43.8% ($p<0.001$) at 10^{-6} M in 2mM glucose. GW9508 was the only GPR120 agonist stimulate GIP secretion in pGIPneo STC-1 cells as it demonstrated a 98.5% increase ($p<0.05$) at 10^{-6} M. Compared to the GPR120 agonist AH7614, GW9508 increased GIP secretion 85.3% ($p<0.05$). ALA and DHA demonstrated no effect on GIP secretion at all concentrations tested (10^{-10} to 10^{-6} M).

In BRIN-BD11 cells, all GPR120 agonists tested demonstrated an insulinotropic effect. The endogenous agonist DHA demonstrated a 1.2 to 2.1-fold increase in insulin secretion ($p<0.01$ to $p<0.001$) at concentrations of 10^{-9} - 10^{-4} M in 5.6mM glucose while ALA increased insulin secretion 1.2-1.5-fold increase ($p<0.05$ - $p<0.001$) at concentrations of 10^{-8} - 10^{-4} M. The synthetic GPR120 agonist GW9508 increased insulin secretion 1.2 to 1.7-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-10} - 10^{-4} M in 5.6mM glucose. The EC_{50} values showed that DHA was the least potent agonist with an EC_{50} value of 3.87×10^{-6} M while the most potent agonist tested in 5.6mM glucose was ALA ($\text{EC}_{50} = 2.04\times 10^{-8}$ M) followed by GW9508 ($\text{EC}_{50} = 5.16\times 10^{-8}$ M).

In 16.7mM glucose, BRIN-BD11 cells treated with ALA had a 1.3 to 1.9-fold ($p<0.05$ - $p<0.001$) insulin in secretion at concentrations of 10^{-8} - 10^{-4} M, DHA increased insulin secretion 1.3 to 1.8-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-9} - 10^{-4} M and GW9508 demonstrated a 1.3 to 1.8-fold ($p<0.05$ - $p<0.001$) increase in insulin secretion at 10^{-9} - 10^{-4} M. GW9508 was the most potent agonist in 16.7mM glucose ($\text{EC}_{50} = 8.26\times 10^{-10}$ M) followed by DHA ($\text{EC}_{50} = 7.34\times 10^{-9}$ M) and ALA ($\text{EC}_{50} = 6.18\times 10^{-8}$ M). No GPR120 agonist had detrimental effects on cell viability.

qPCR confirmed the expression of GPR120 in the small intestine while double immunohistochemistry confirmed the co-localisation of GPR120 with GLP-1, GIP and PYY. HFF NIH Swiss mice demonstrated a 29.1% ($p<0.05$) downregulation of GPR120 in the small intestine when compared to lean mice. Treatment of GLUTag cells with GPR120 demonstrated an upregulation compared to untreated cells. ALA increased GPR120 expression 4.6-fold ($p<0.01$) compared to untreated cells while GW9508 increased proglucagon expression 4.7-fold ($p<0.01$). There was no difference between these agonists and basal control. DHA increased GPR120 gene expression 2.6-fold compared to untreated GLUTag cells however this was a 52.7% downregulation compared to 2mM glucose ($p<0.01$).

Proglucagon gene expression was increased in GLUTag cells treated by ALA and DHA compared to vehicle control. DHA treatment resulted in a 29.5% ($p<0.05$) upregulation when compared to untreated cells which increased to a 2.4-fold ($p<0.01$) increase compared to vehicle control. ALA increased proglucagon gene expression 2.0-fold ($p<0.01$) compared to 2mM glucose with no difference to untreated cells. GW9508 had no effect on proglucagon gene expression in GLUTag cells compared to 2mM glucose, however a 42.7% ($p<0.05$) downregulation was observed compared to untreated cells.

As the proglucagon gene is cleaved by PC1/3 to release GLP-1, the expression of this gene was also investigated. Interestingly treatment of agonists completely ablated PC1/3 gene expression ($p<0.001$).

Acute oral administration of GPR120 agonists both alone and in combination with Sitagliptin in lean Swiss TO mice ($n=6$) reduced blood glucose and increased insulin secretion. Oral administration of DHA caused a 22.2% ($p<0.05$) in blood glucose. When DHA was administered alongside the GPR120 antagonist AH7614, there was a 20.3% ($p<0.05$) increase in blood glucose compared to DHA administration alone. DHA administration in combination with Sitagliptin reduced blood glucose 52.1% overall ($p<0.001$). Administration of GW9508 decreased blood glucose 31.3% ($p<0.05$) while combining GW9508 with Sitagliptin resulted in a 34.0% ($p<0.01$) overall decrease.

Oral administration of DHA increased insulin secretion from male Swiss TO mice 1.9-fold ($p<0.001$) while a combination of DHA and Sitagliptin resulted in a 1.7-fold ($p<0.001$) increase. When DHA administration is compared to DHA combined with AH7614, DHA administration causes a 1.6-fold ($p<0.001$) increase in insulin secretion. GW9508 administration increases insulin secretion 1.8-fold ($p<0.001$) and in combination with Sitagliptin a 1.9-fold ($p<0.001$) increase is observed. GW9508 also increases insulin secretion 1.5-fold ($p<0.05$) compared to GW9508 in combination with Sitagliptin.

Oral administration of GPR120 agonists also increased incretin hormone secretion in male Swiss TO mice. DHA increased GLP-1 secretion 2.7-fold ($p<0.001$) 30 mins post administration while this was a 3.3-fold ($P<0.001$) increase in mice treated with GW9508. Both agonists also increased GIP secretion with increases of 2.6-fold ($p<0.001$) and 2.7-fold ($p<0.001$) observed 15 mins post administration for DHA and GW9508 respectively. Further to this both agonists in combination with Sitagliptin reduced appetite with decreases of 16.9% ($p<0.05$) and 19.8% ($p<0.05$) observed respectively in Swiss TO mice trained to eat for 3 hrs ($n=8$).

The results of this study further establish a role for GPR120 activation in incretin and gastrointestinal hormone secretion and may be an important therapeutic target for in the treatment of Type 2 Diabetes and metabolic disorders.

5.2: Introduction

GPR120 is a 377-amino acid long rhodopsin-like GPCR that was first recognised as being present on chromosome 10 in 2003 using bioinformatical analysis of the human genome (Fredriksson *et al.* 2003a). This study found the human GPR120 gene which was expressed in the stomach and had 4 coding exons. The sequence for the GPR120 gene was highly conserved in mice with 86% similarity, however the mouse protein is 16 amino acid residues shorter at 361 amino acids and present on chromosome 19 (Fredriksson *et al.* 2003a). As no ortholog was found within fish it was determined that, along with GPR141, GPR120 must have evolved into the mammalian genome at a stage after it had diverged from the common mammalian ancestor with fish (Fredriksson *et al.* 2003a). It was later found that human GPR120 has both the long isoform reported above and a shorter isoform consisting of 361 amino acids (Strausberg *et al.* 2002).

As an orphan receptor, GPR120 was of interest to researchers looking for novel treatments for a range of diseases. One of the first disorders which was of interest to researchers was Type 2 Diabetes (Hirasawa *et al.* 2005a). Using qPCR, it was found that GPR120 was expressed in both human and mouse small intestine as well as the intestinal STC-1 cell line which is also derived from mice. Interestingly this study did not find GPR120 to be present in a range of other cell lines including the pancreatic MIN6 cell line (Hirasawa *et al.* 2005a). It was discovered that a range of long chain fatty acids such as palmitoleic, alpha-linoleic (ALA) and docosahexaenoic acid (DHA) all stimulated GPR120 (Hirasawa *et al.* 2005a). Saturated fatty acids stimulated GPR120 in shorter chains (C14 to C18) than unsaturated fatty acids (C16 to C22). As the free form of fatty acids had previously been shown to increase both CCK (Guimbaud *et al.* 1997) and GLP-1 in humans (Holst, Orskov 2001), the research then focused on the effects of these fatty acids on GLP-1 secretion from STC-1 cells through GPR120. Using siRNAs, the researchers were able to determine that the fatty acids used were able to stimulate GLP-1 secretion through GPR120 but not GPR40 (Hirasawa *et*

al. 2005a). GPR40 was also targeted in this research as it is also a free fatty acid receptor that is activated by long chain fatty acids (Watson, Brown & Holliday 2012). Despite both receptors being activated by long chain fatty acids, they share only 10% sequence homology (Hirasawa *et al.* 2008). However due to the similarity in their agonists, they are classified as free fatty acid receptors alongside GPR41 and GPR43 (Hirasawa *et al.* 2008, Stoddart, Smith & Milligan 2008).

While long chain fatty acids activate GPR120, some can bind to the receptor and prevent ligand binding, resulting in an antagonistic effect. AH7614 is a GPR120 specific antagonist which can be used to block the activation of the receptor (Sparks *et al.* 2014). AH7614 is able to antagonise GPR120 as it is a negative allosteric modulator that decreases the affinity of GPR120 agonists for the receptor (Watterson *et al.* 2017). Another possible GPR120 antagonist known as TUG-1837 may be able to exert the same effects as AH7614 via the same mechanism (Watterson *et al.* 2017).

The second hormone linked with metabolic disorders to be investigated was CCK (Tanaka *et al.* 2008b). This *in vivo* study determined that the GPR120 agonists ALA, palmitoleic and octanoic acid all increased CCK secretion from intestinal L-cells in a GPR120 specific manner. In order to confirm the specificity of these agonists for GPR120, shRNAs were used to block the action of either GPR120 or GPR40. GPR40 was targeted in both studies as it is also activated by medium to short chain fatty acids (Itoh *et al.* 2003a).

As CCK has previously been shown to increase insulin secretion (Rushakoff *et al.* 1987b) and GLP-1 has a well defined incretin effect on insulin secretion, further research focused on the presence of GPR120 and effects of GPR120 agonists on insulin secretion from the pancreas. Contrary to the earlier studies (Hirasawa *et al.* 2005a), GPR120 was found to be present in both pancreatic BRIN-BD11 and INS1 cell lines (Dhayal, Welters & Morgan 2008) as well as the MIN6 cell line which it was previously reported to be absent from as well as isolated mouse islets (Kebede *et al.* 2009). The presence of GPR120 was confirmed in the BRIN-BD11 cell line as well as isolated islets in a study which investigated both the *in vitro* and *in vivo* effects of GPR120 agonists (Moran *et al.* 2014). This study identified that activation of GPR120 agonists increased insulin secretion from BRIN-BD11 cells while having glucose lowering effects *in vivo* in lean mice. It was suggested that this was through both the Ca^{2+} and cAMP pathway, however this needs further study as previous research did not find any cAMP pathway activation upon GPR120 stimulation, rather it was found that GPR120 increased intracellular calcium and activated the ERK signalling cascade (Hara *et al.* 2011). This research suggests that GPR120 binds to the $\text{G}\alpha_q$ family, however further investigation in our lab has confirmed that GPR120 agonists have an effect on cAMP signalling (unpublished data). This further work has also examined the effects of GPR120 activation on glucagon secretion as it has been suggested that GPR120 agonists have a role to play in glucagon secretion in the pancreatic islet (Suckow *et al.* 2014). Another pancreatic hormone of interest is somatostatin which

was studied using knock-in mice. The authors of this study demonstrated the co-localisation of GPR120 with somatostatin in the delta cells of the islet as well as the loss of response in δ -cells of GPR120 knockout mice (Stone *et al.* 2014).

The most recent hormone which has been studied in relation to the effect of GPR120 on diabetes is GIP (Iwasaki *et al.* 2015). This research used GFP knock-in mice to try and identify the K cells of the intestine whilst also looking at the expression of fatty acid receptors. The presence of GPR120 in GFP positive K cells was identified and confirmed using GPR120 knockout mice. It was found that GPR120 knockout reduced GIP secretion and increased blood glucose.

Further research into the role of GPR120 focused on obesity, which is closely linked to Type 2 Diabetes, as well as a range of other disorders. In humans of European descent, a mutation (p.R270H) was identified in the GPR120 gene of obese subjects that inhibited GPR120 signalling (Ichimura *et al.* 2012). Furthermore, mice without GPR120 which were fed a high fat diet developed obesity, fatty livers and glucose intolerance (Ichimura *et al.* 2012). GPR120 has also been implicated in reducing inflammation (Oh *et al.* 2010) which is one of the many pathophysiological effects of Type 2 Diabetes.

All of the above research implicates GPR120 in an anti-diabetic role. More research is necessary to understand the role of GPR120 activation in intestinal hormone secretion and glucose homeostasis. The effects of GPR120 activation on GLP-1 and GIP were investigated in GLUTag and pGIPneo STC-1 cell lines, while the insulinotropic actions of GPR120 agonists ALA, DHA and GW9508 were studied in pancreatic BRIN-BD11 and intestinal. Gene and protein expression of GPR120 was investigated using qPCR and histological analysis. The acute biological effects of GPR120 agonists, DHA and GW9508, were assessed in male Swiss TO mice.

5.3: Materials and methods

5.3.1: Materials

ALA (L2376-500MG) was purchased from Sigma-Aldrich (Poole, Dorset, UK) whilst DHA (ab145212), GW9508 (ab144444) and AH7614 (ab146181) were purchased from Abcam (Cambridge, UK). Multi species GLP-1 total and rat/mouse GIP total ELISA kits were purchased from Merck Millipore (Watford, UK).

5.3.2: Acute insulin secretion from pancreatic BRIN-BD11 cells

Cells were seeded in 24-well plates with 150000 BRIN-BD11 cells per well and incubated overnight in 1ml of RPMI 1640 media at 37°C and 5% CO₂. Following this incubation, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹² to 10⁻⁴M concentrations of ALA, DHA and GW9508 with 10mM alanine as a positive control. After incubations, supernatants (950µl) were collected and frozen at -20°C until radioimmunoassay could be carried out as described in Chapter 2, section 2.2.

5.3.3: Acute hormone secretion from intestinal cell lines

GLUTag or pGIPneo STC-1 cells were seeded into 24-well plates with 150000 cells per well which were incubated overnight in 1ml of culture medium (as described previously) at 37°C and 5% CO₂. To ensure that cells grew in monolayers, they were pipetted up and down vigorously to ensure proper mixing before seeding. After overnight incubation and preincubation with 1.1mM glucose for 40 mins, acute incubations were carried out at 37°C 5% CO₂ for 2 hrs in 2mM glucose in KRBB buffer supplemented with 10⁻¹⁰ to 10⁻⁴M concentrations of ALA, DHA and GW9508. Test solutions were added both alone and in combination with the GPR120 antagonist AH7614 at a concentration of 10⁻⁵M. After incubations, supernatants (950µl) were collected and frozen at -20°C until ELISA could be carried out as described in Chapter 2, section 2.3.

5.3.4: Acute effects of ALA, DHA and GW9508 on cell toxicity

Cells were seeded in 96-well plates with 40000 cells per well of BRIN-BD11, GLUTag or pGIPneo STC-1 cells and incubated overnight in cell culture media (as described in Chapter 2, section 2.1) in an atmosphere of 5% CO₂ at 37°C. After 20hrs, BRIN-BD11 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of GPR120 agonist with 1mM H₂O₂ as a cytotoxic control (n=3). After 48hrs, GLUTag and pGIPneo STC-1 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 2 hrs in 2mM in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of ALA, DHA and GW9508 with 1mM H₂O₂ as a cytotoxic control (n=3). After incubation, supernatant was decanted, cells were washed with HBSS and 0.5mg/ml MTT was added for 2 hrs. Following MTT incubation, supernatant was discarded and DMSO was added to solubilise crystals. Optical density was subsequently measured at 570nm and 630nm using the Flexstation 3 (Molecular Devices, CA, USA).

5.3.5: Immunofluorescence staining in GLUTag, pGIPneo STC-1, STC-1 cells and lean and HFF NIH Swiss mouse small intestine

GLUTag, pGIPneo STC-1 and STC-1 cells were seeded at a density of 40000 cells on to polylysine coated slides overnight at 37°C in an atmosphere of 95% air and 5% CO₂ as described in Chapter 2, section 2.1. Swiss TO mice were anaesthetised by isoflurane and killed by cervical dislocation. Pancreas and intestine was excised as described in Chapter 2, section 2.6.3. Immunohistochemistry was carried out by incubating with rabbit polyclonal anti-GPR120 and goat polyclonal anti-GLP-1, goat polyclonal anti-GIP or goat polyclonal anti-PYY with the dilutions outlined in Chapter 2, Table 2.1.

5.3.6: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Chapter 2, Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH.

5.3.7: Acute *in vivo* glucose tolerance tests

Male Swiss TO mice were housed as described in Chapter 2, section 2.7. The effects GPR120 agonists DHA and GW9508 (0.1µmol/kg BW), as well as in combination with Sitagliptin Phosphate (50mg/kg BW) in lean male Swiss TO mice were investigated. Before test compounds were administered mice were fasted for 18 hrs and blood subsequently collected (t=0). Test compounds were administered orally in glucose (18mmol/kg BW), and blood was collected at 15, 30, 60, 90 and 120 mins along with blood glucose measurements. Collected blood was centrifuged at 13200rpm for 5 mins at 4°C using a Beckman centrifuge (Beckman Instruments, UK). Plasma was stored at -20°C until hormone measurements by RIA or ELISA as described in Chapter 2, sections 2.2 and 2.3.

5.4: Results

5.4.1: Effects of GPR120 agonists and antagonist AH7614 on GLP-1 secretion from intestinal GLUTag cells

The effects of endogenous GPR120 agonists ALA and DHA and synthetic agonist GW9508 along with GPR120 antagonist AH7614 on GLP-1 secretion from GLUTag cells in 2mM glucose was examined. The endogenous GPR120 agonist DHA (Figure 5.2A) had the greatest increase in GLP-1 secretion as it demonstrated a 1.6 to 2.7-fold ($p<0.01$ to $p<0.001$) increase compared to basal levels at 10^{-8} to 10^{-6} M. This was followed by the endogenous agonist ALA (Figure 5.1A) which exhibited a 1.5 to 1.8-fold increase ($p<0.01$) from 10^{-8} - 10^{-6} M and the synthetic agonist GW9508 (Figure 5.3A) which increased GLP-1 secretion 1.8-fold ($p<0.001$) from GLUTag cells at 10^{-6} M in 2mM glucose.

When GLUTag cells were treated with both a GPR120 agonist and the antagonist AH7614 (10^{-5} M) all agonists tested demonstrated selectivity for the GPR120 receptor as AH7614 reduced the agonistic action on GLP-1 secretion ($p<0.05$ - $p<0.01$). In the presence of AH7614, ALA secretory action was reduced from 1.3 to 1.5-fold (10^{-8} M to 10^{-6} M, $p<0.05$ - $p<0.01$) while DHA action was reduced 1.7 to 2.4-fold (10^{-10} M to 10^{-6} M, $p<0.01$). The secretory ability of synthetic GPR120 agonist GW9508 was reduced 1.5 to 1.7-fold (10^{-8} M to 10^{-6} M, $p<0.01$). All agonists tested demonstrated no adverse effects on cell viability or cell proliferation as observed through measurement of MTT (Figures 5.1B, 5.2B and 5.3B).

5.4.2: Effects of GPR120 agonists and antagonist AH7614 on GIP secretion from intestinal pGIPneo STC-1 cells

The effects of GPR120 agonists on GIP secretion was investigated through treatment of intestinal pGIPneo STC-1 cells. The endogenous agonists tested (ALA and DHA) had no significant effect on GIP secretion compared to vehicle control (2mM glucose) (Figures 5.4A and 5.5A). Synthetic GPR120 agonist GW9508 (Figure 5.6A) demonstrated a 2.0-fold ($p<0.05$) increase in GIP secretion from pGIPneo STC-1 cells at 10^{-4} M. Treatment of pGIPneo STC-1 cells with both GPR120 agonist GW9508 (10^{-4} M) and antagonist AH7614 (10^{-5} M) reduced GIP secretion to basal levels (1.9-fold decrease) demonstrating the selectivity of GW9508 for GPR120. All agonists tested demonstrated no effects on cell viability or cell proliferation (Figures 5.4B, 5.5B and 5.6B).

5.4.3: Effects of GPR120 agonists on insulin secretion from pancreatic BRIN-BD11 cells

The effects of both endogenous (ALA and DHA) and synthetic (GW9508) fatty acid GPR120 agonists on insulin secretion were investigated in the clonal BRIN BD11 cell line in both normal and hyperglycaemic conditions (Figures 5.7-5.12). The endogenous agonist DHA (Figure 5.9A) demonstrated a 1.2 to 2.1-fold increase in insulin secretion ($p<0.01$ - $p<0.001$) at concentrations of 10^{-9} - 10^{-4} M in 5.6mM glucose. ALA (Figure 5.7A) increased insulin secretion 1.2 to 1.5-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-8} - 10^{-4} M in 5.6mM glucose while the synthetic GPR120 agonist GW9508 (Figure 5.11A) increased insulin secretion 1.2 to 1.7-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-1} to 10^{-4} M at the same concentration of glucose. DHA was the least potent agonist examined in 5.6mM glucose (Figure 5.13A) with an EC_{50} value of 3.87×10^{-6} M. The most potent agonist tested in normal conditions was ALA which had an EC_{50} value of 2.04×10^{-8} M which was closely followed by GW9508 (5.16×10^{-8} M).

When examining the insulintropic effects of GPR120 agonists in hyperglycaemic conditions, all agonists demonstrated similar effects. ALA (Figure 5.8A) increased insulin secretion 1.3 to 1.9-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-8} - 10^{-4} M. The endogenous agonist DHA (Figure 5.10A) demonstrated a 1.3 to 1.8-fold ($p<0.05$ - $p<0.001$) increase in insulin secretion at 10^{-9} - 10^{-4} M. The synthetic agonist GW9508 (Figure 5.12A) exhibited a 1.3 to 1.8-fold ($p<0.05$ - $p<0.001$) insulintropic effect in 16.7mM glucose at concentrations of 10^{-9} to 10^{-4} M. The most potent agonist tested in hyperglycaemic conditions was GW9508 (EC_{50} 8.26×10^{-10} M). This was followed by DHA (EC_{50} 7.34×10^{-9} M) with ALA (EC_{50} 6.18×10^{-8} M) being the least potent agonist (Figure 5.13B). There were no adverse effects on cell viability or proliferation (Figures 5.7B-5.12B) at either normal or hyperglycaemic conditions for all GPR120 agonists tested.

5.4.4: Distribution of GPR120, GLP-1, GIP and PYY in intestinal cell lines and lean and HFF NIH Swiss mouse small intestine

The presence or absence of GPR120 in the L and K cells of the intestine was determined through double immunohistochemistry in both lean and HFF diet mice as well as GLUTag, pGIPneo STC-1 and STC-1 cells. Both GPR120 and GLP-1 were expressed throughout GLUTag cells (Figure 5.14). Immunohistochemistry in both lean and HFF small intestine confirmed the co-localisation of GPR120 and GLP-1 in intestinal L-cells (Figure 5.15D and 5.16D). PYY, also secreted from the L-cells of the small intestine, was found to be co-localised with GPR120 in both STC-1 cells (Figure 5.21) and lean and HFF mouse small intestine (Figure 5.22D and 5.23D) thus confirming the presence of GPR120 in L-cells. GIP, secreted from intestinal K-cells, was observed to be co-localised with GPR120 in lean (Figure 5.19D) and HFF (Figure 5.20D) diet mouse small intestine as well as pGIPneo STC-1 cells (Figure 5.18D). qPCR was carried out on whole lean and HFF diet

mouse small intestine (Figure 5.17) to determine how diet affects GPR120 expression. It was determined that mice fed a HFF diet have a 1.4-fold reduction in intestinal GPR120 expression ($p < 0.05$). However this result confirmed that seen in GPR120 staining with GPR120 being present in both lean and HFF mouse small intestinal tissue.

5.4.5: Effects of GPR120 agonists on proglucagon, PC1 and GPR120 gene expression in intestinal GLUTag cells

The effects of GPR120 agonists in 2mM glucose on GPR120 gene expression was determined using qPCR (Figure 5.24A). Treatment of GLUTag cells with DHA demonstrated a 2.1-fold decrease in GPR120 gene expression compared to vehicle control ($p < 0.01$). GPR120 agonists ALA and GW9508 had no significant effect on expression compared to 2mM glucose vehicle control (Figure 5.24A).

DHA was the GPR120 agonist with the greatest increase on proglucagon gene expression in GLUTag cells (Figure 5.24B) with a 2.4-fold ($p < 0.01$) increase compared to vehicle control. Compared to the vehicle control, ALA exhibited a 2.0-fold increase ($p < 0.01$) in proglucagon gene expression. GW9508 had no effect compared to vehicle control.

As the proglucagon gene is cleaved by PC1/3 to release GLP-1, the expression of this gene was also investigated. Interestingly treatment of agonists completely ablated PC1/3 gene expression ($p < 0.001$, Figure 5.24C).

5.4.6: Acute effects of GPR120 agonists on incretin hormones in male Swiss TO mice

The effects of oral administration of GPR120 agonists on the incretin hormones GLP-1 and GIP was also investigated in this study. Both DHA and GW9508 increased the secretion of these hormones. DHA (Figure 5.25A) increased GLP-1 secretion 2.7-fold ($p < 0.001$) 30 mins post administration when compared to glucose administration. GW9508 (Figure 5.25A) had a more sustained effect, with increases of 2.1 and 3.2-fold ($p < 0.001$) being observed 15 and 30 mins post administration respectively.

As seen with GLP-1, both agonists tested demonstrated increases in GIP secretion (Figure 5.26A). DHA exhibited a 2.6-fold ($p < 0.001$) increase in GIP secretion whilst GW9508 demonstrated a 2.7-fold ($p < 0.001$) increase.

5.4.7: Acute effects of GPR120 agonists on blood glucose and insulin in male Swiss TO mice

Oral administration of GPR120 agonists DHA (Figure 5.27B) and GW9508 (Figure 5.28B) at concentrations of 0.1 $\mu\text{mol/kg}$ body weight in fasted Swiss TO mice reduced blood glucose 22% ($p<0.05$) and 31% ($p<0.05$) over 120 mins with synthetic GPR120 agonist GW9508 demonstrating the most potent properties.

GW9508 had reduced blood glucose 25% ($p<0.05$) 15 mins post administration with this effect continuing 30 mins post administration with a 36% ($p<0.001$) reduction in blood glucose (Figure 5.28A). Administration of GW9508 with GPR120 antagonist AH7614 confirmed the selectivity of GW9508 for GPR120 with reductions in blood glucose of 37% ($p<0.01$), 32% ($p<0.05$) and 29% ($p<0.01$) after 30, 60 and 90 mins post administration respectively.

DHA (Figure 5.27A) reduced blood glucose 27% ($p<0.001$) and 30% ($p<0.001$) after 15 and 30 mins post administration. As observed with GW9508, administration of DHA with GPR120 antagonist AH7614 confirmed its selectivity for GPR120 with DHA administration alone decreasing blood sugar 26% ($p<0.001$) and 18% ($p<0.05$) after 15 and 30 mins, respectively.

Administration of DHA with DPP-IV inhibitor Sitagliptin Phosphate (Figure 5.27A) demonstrated a marked reduction in blood glucose compared to both vehicle control and DHA itself. Compared to glucose, blood glucose was reduced 51% ($p<0.001$), 63% ($p<0.001$), 54% ($p<0.001$), 51% ($p<0.001$) and 52% ($p<0.001$) at 15, 30, 60, 90 and 120 mins post administration respectively. Compared to DHA itself, DHA in combination with Sitagliptin reduced blood glucose 33% ($p<0.001$), 49% ($p<0.001$), 43% ($p<0.001$), 36% ($p<0.01$) and 34% ($p<0.05$) at 15, 30, 60, 90 and 120 mins post administration. When GW9508 was administered in conjunction with Sitagliptin (Figure 5.28A), blood glucose was decreased 34% ($p<0.001$) and 41% ($p<0.001$) after 15 and 30mins. There was no significant difference between GW9508 combination treatment and GW9508 alone.

Oral administration of GPR120 agonists also lead to increased insulin secretion. DHA treatment alone (Figure 5.29A) lead to a 3.4-fold increase 30 mins post administration compared to glucose control ($p<0.001$). The same effect was seen when DHA was administered in combination with Sitagliptin with a 3.09-fold increase being observed at this time point ($p<0.001$). Sitagliptin treatment alone lead to a 2.1-fold increase in insulin secretion 15 mins post administration ($p<0.001$), however this effect was not sustained after this. The insulinotropic effect of DHA was reduced 2.2-fold when administered in the presence of GPR120 antagonist AH7614 (0.1 $\mu\text{mol/kg}$ body weight, $p<0.001$).

Administration of GW9508 (Figure 5.30A) led to a 2.6-fold increase in insulin secretion ($p<0.001$). Like DHA this effect was seen 30 mins post administration. GW9508 in combination with

Sitagliptin had a greater insulinotropic effect than GW9508 treatment alone with an increase of 2.8-fold being observed 30 mins post administration ($p<0.001$). AH7614 also reduced the insulinotropic effects of GW9508 with a decrease of 1.6-fold being exhibited.

5.4.8: Acute effects of GPR120 agonists on feeding

The acute effects of oral administration of GPR120 agonists on male Swiss TO mice trained to eat for 3 hrs only was investigated. DHA (Figure 5.31A) had an initial effect on appetite with feeding being reduced 33% ($p<0.01$) and 22% ($p<0.05$) after 30 mins and 1 hr respectively. However this effect was not sustained and there was no difference in total feeding over 3 hrs when compared to saline control. When DHA was administered in combination with Sitagliptin, appetite was reduced after 150 and 180 mins by 18% ($p<0.01$) and 17% ($p<0.05$) respectively.

Oral administration of GW9508 (Figure 5.31B) also resulted in an initial reduction in appetite as this was reduced for the first 90 mins with reductions of 65% ($p<0.001$), 49% ($p<0.001$), 38% ($p<0.001$), 25% ($p<0.05$) and 21% ($p<0.05$). Like DHA, this effect was not sustained and after 3 hrs of feeding there was no effect. However, administration of a combination of GW9508 and Sitagliptin did result in an overall reduction in appetite with an appetite reduction of 20% ($p<0.05$) after 3hrs.

5.5: Discussion

GPR120 has previously been shown to play a role in incretin (Hirasawa *et al.* 2004, Iwasaki *et al.* 2015) and other gastrointestinal hormone (Tanaka *et al.* 2008) secretion. GPR120 is activated by long chain omega 3 fatty acids (Hirasawa *et al.* 2004) and a mutation in GPR120 has shown increased levels of obesity in humans of European descent (Ichimura *et al.* 2012). This has led to interest into how GPR120 activation can be used as a therapeutic treatment in metabolic disorders such as Type 2 Diabetes (Moran *et al.* 2014).

This study investigated the effects of both endogenous and synthetic GPR120 agonists on intestinal hormone and insulin secretion. ALA, DHA and GW9508 effects on GLP-1 secretion were assessed in GLUTag cells. The agonist which caused the greatest increase in GLP-1 secretion was DHA followed by GW9508 then ALA. Both DHA and ALA have previously been shown to increase GLP-1 secretion from STC-1 cells (Hirasawa *et al.* 2005a). GLP-1 secretion was increased in a dose dependent manner with agonist specificity being confirmed through AH7614 antagonism. When assessing GIP secretion from pGIPneo STC-1 cells, only the synthetic agonist GW9508 caused an increase. The omega 3 fatty acids ALA and DHA had no effect on GIP secretion. These results

confirm previous research (Hirasawa *et. al.* 2004, Iwasaki *et. al.* 2015) which suggested that GPR120 may play a role in incretin hormone secretion. All agonists tested did not cause any cytotoxicity in the cell lines tested. The effects of GPR120 agonists on insulin secretion were investigated in BRIN-BD11 cells. In normoglycaemic conditions ALA performed most efficiently followed by GW9508 and DHA. In hyperglycaemic conditions GW9508 was the most efficient agonist followed by DHA then ALA. These results are similar to those previously reported (Moran *et. al.* 2014).

Previous research has indicated the presence of GPR120 in both the L-cells (Hirasawa *et. al.* 2004) and K-cells (Iwasaki *et. al.* 2015) of the intestine. In order to confirm these findings, the expression of GPR120 in the small intestine was determined via qPCR and double immunohistochemistry. As qPCR was carried out in whole intestine extract, the double immunohistochemistry was used to confirm the cell types which GPR120 is present in. GIP is secreted from K cells of the intestine and staining of both pGIPneo STC-1 cells and lean and HFF small intestine confirmed the presence of GPR120 in GIP secreting K cells. Both PYY and GLP-1 are secreted from the L cells of the intestine and double immunohistochemistry in lean and HFF mouse small intestine as well as GLUTag cells for GLP-1 and STC-1 cells for PYY confirmed the presence of GPR120 in the L cells of the intestine. Mice fed a high fat diet had reduced GPR120 expression which is in keeping with recent research which found various changes in GPR120 expression in the gastrointestinal tract when mice were fed a high fat diet (Widmayer *et. al.* 2015). GPR120 was upregulated in GLUTag cells when cells were treated with 2mM glucose however neither ALA nor GW9508 had any effect. DHA downregulated GPR120 expression yet upregulated proglucagon gene expression suggesting that GPR120 was being internalised and the downstream signalling events occurring due to receptor internalisation are increasing proglucagon production. This has previously been seen for GPR120 when activated by GW9508 in human eosinophils (Konno *et al.* 2015). ALA also upregulated proglucagon gene expression suggesting that the production of GLP-1 was being increased. PC1/3 expression would be expected to increase in tandem with proglucagon gene expression, however as these agonists increase GLP-1 secretion the cellular stores of proglucagon may need replenished and PC1/3 production halts. However further research is needed to confirm this.

As DHA and GW9508 increased GLP-1 to a greater degree than that demonstrated by ALA and that GW9508 was the only agonist to increase GIP secretion, both DHA and GW9508 were selected for further investigation. Furthermore ALA has also been previously shown to have no effect on GLP-1 secretion (Paulsen *et. al.* 2014) and DHA provided the greatest increase in GLP-1 secretion and may have increased GIP secretion in a GPR120 specific manner at higher concentrations *in vitro*. ALA demonstrated no trend compared to GPR120 antagonism and was not selected for further study for these reasons. In order to determine the effects of these agonists on gastrointestinal hormone secretion, they were administered by oral gavage rather than intraperitoneal injection. Both

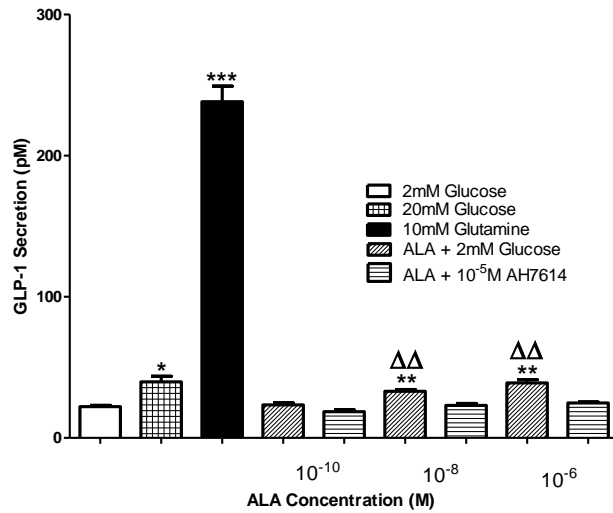
agonists increased insulin secretion and reduced blood glucose. This has been demonstrated previously via IP injection (Moran *et al.* 2014b, Shimura *et al.* 1997) or intracolonicly (Shida *et al.* 2013). Oral administration of DHA has shown to improve arthritis in the knee (Torres-Guzman *et al.* 2014) thus suggesting that it may also have anti-inflammatory effects. The oral effects of GW9508 have not yet been investigated. Both agonists were administered alongside the DPP-IV inhibitor Sitagliptin and demonstrated an additive effect in the reduction of blood glucose.

Intracolonic administration of DHA has previously shown that DHA administration can increase GLP-1 secretion (Emery *et al.* 2014), however this is the first study to determine an increase through oral administration. GW9508 also demonstrated the ability to increase GLP-1 and this effect was demonstrated for a longer time period than DHA. There is currently no published research determining the effects of DHA and GW9508 on GIP secretion. In this study both GIP and DHA increased GIP secretion when administered orally *in vivo*. Both agonists demonstrated similar effects with a prompt increase in GIP after 15 mins and returning to basal levels by 30 min. GLP-1 has demonstrated an ability to slow gastric emptying (Shah, Vella 2014), while the effects of GIP on satiety appear to be minimal (Edholm *et al.* 2010b). Despite an initial reduction in appetite, agonist treatment alone did not reduce appetite. However in combination with Sitagliptin the satiety effect was prolonged and appetite was reduced.

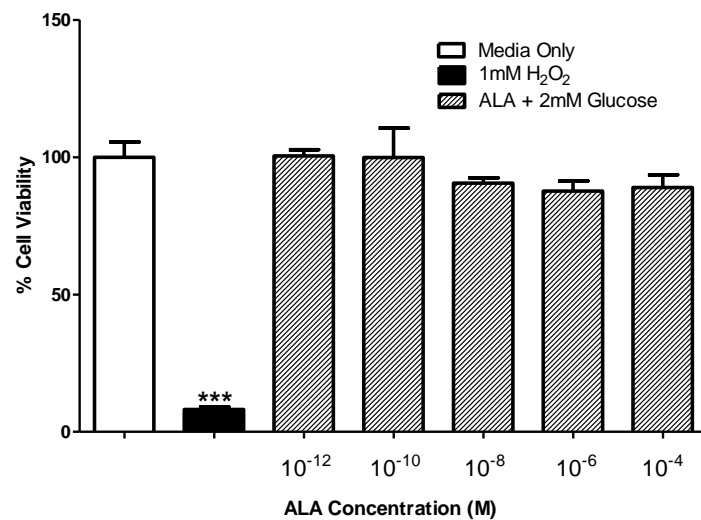
In conclusion, this study is the first study to identify the GIP GLP-1 secreting effects of DHA and GW9508 *in vitro* and orally *in vivo*. Administration in combination with Sitagliptin also reduced appetite *in vivo*. GPR120 expression was confirmed in a range of intestinal cell lines as well as lean and HFF mouse small intestine. As well as increasing GLP-1 and GIP, oral administration of DHA and GW9508 reduces blood glucose and increase plasma insulin. The results presented in this study demonstrate a role for GPR120 agonists in glucose homeostasis through intestinal hormone secretion and they may be a potential therapy in the treatment of metabolic disorders such as Type 2 Diabetes.

Figure 5.1: Effects of endogenous GPR120 agonist ALA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose

A.



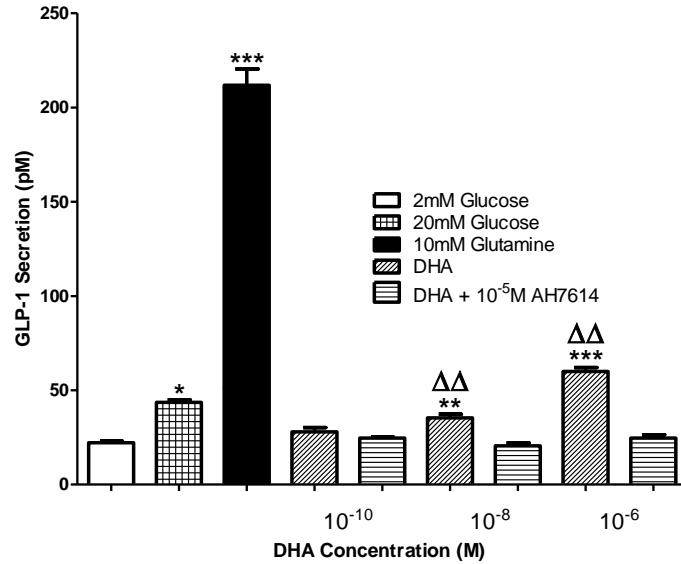
B.



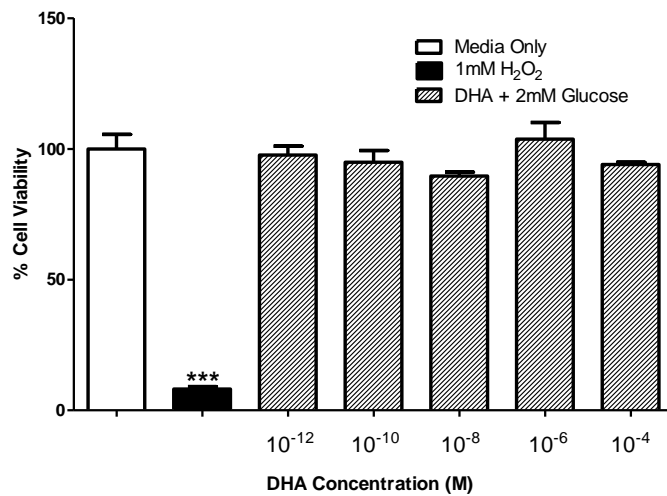
Effect of GPR120 agonist ALA (10^{-8} - 10^{-4} M) and GPR120 antagonist AH7614 (10^{-5} M) on GLP-1 secretion (A) and cell viability (C) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ $p < 0.05$ and $\Delta\Delta$ $p < 0.01$ compared to antagonist treatment at same concentration of agonist.

Figure 5.2: Effects of DHA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose

A.



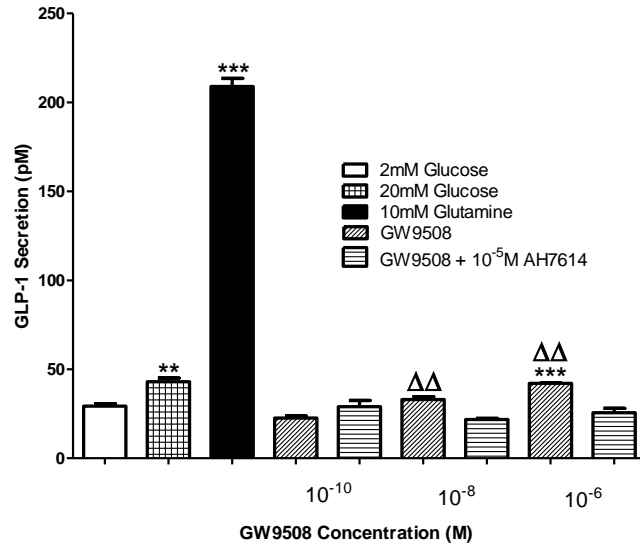
B.



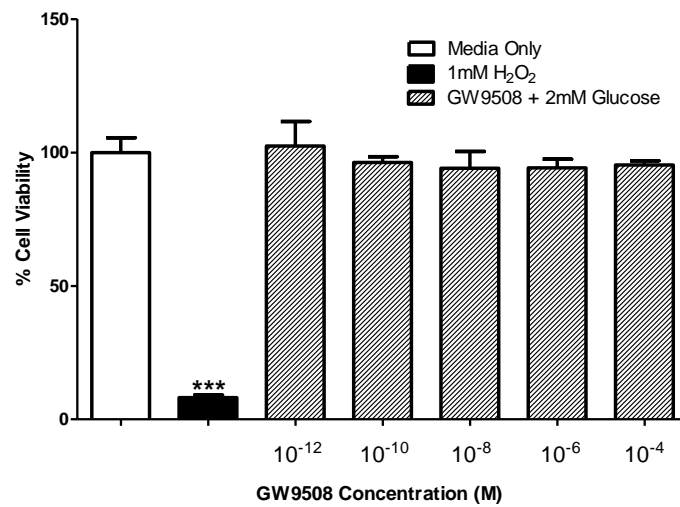
Effect of GPR120 agonist DHA (10^{-10} - 10^{-6} M) and antagonist AH7614 (10^{-5} M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. $\Delta\Delta$ $p < 0.01$ compared to antagonist treatment at same concentration of agonist.

Figure 5.3: Effects of GW9508 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose

A.

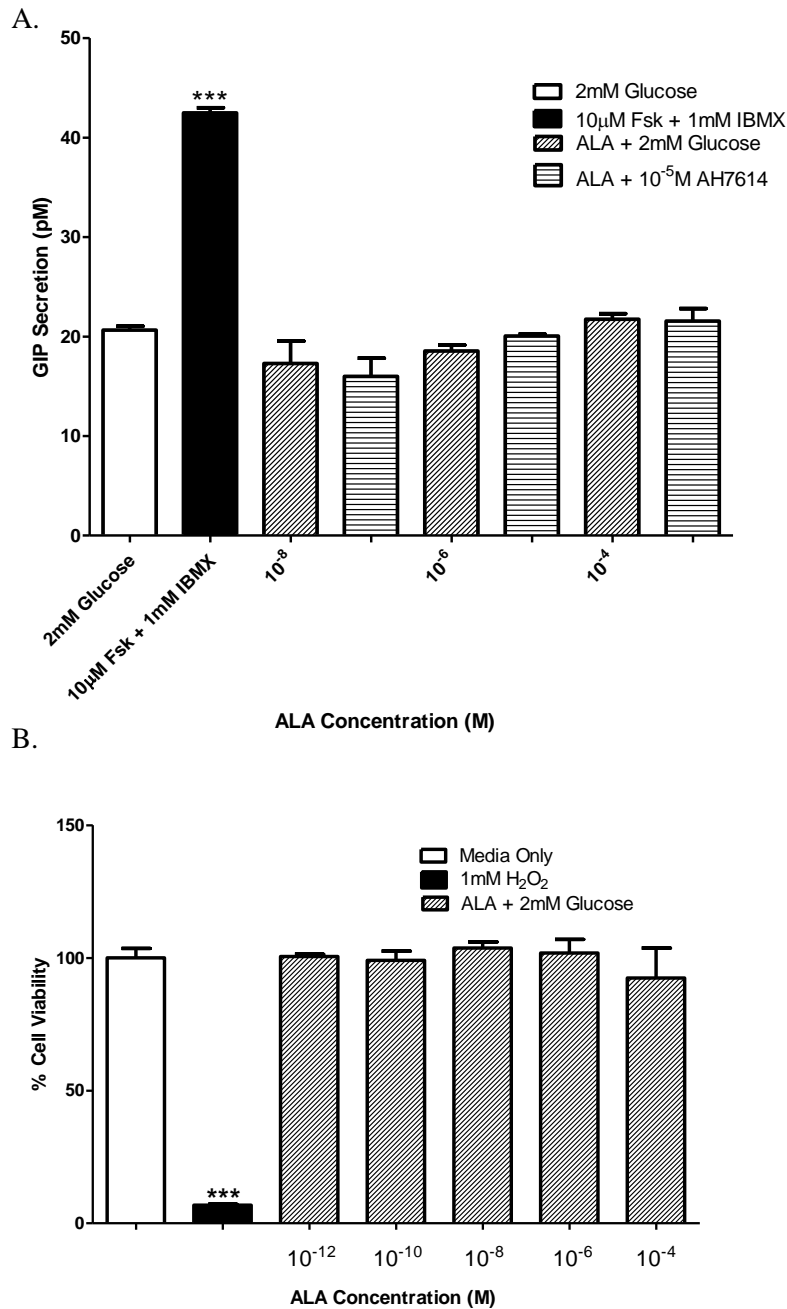


B.



Effect of GPR120 agonist GW9508 (10^{-10} - 10^{-6} M) and antagonist AH7614 (10^{-5} M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. $\Delta\Delta$ $p < 0.01$ compared to antagonist treatment at same concentration of agonist.

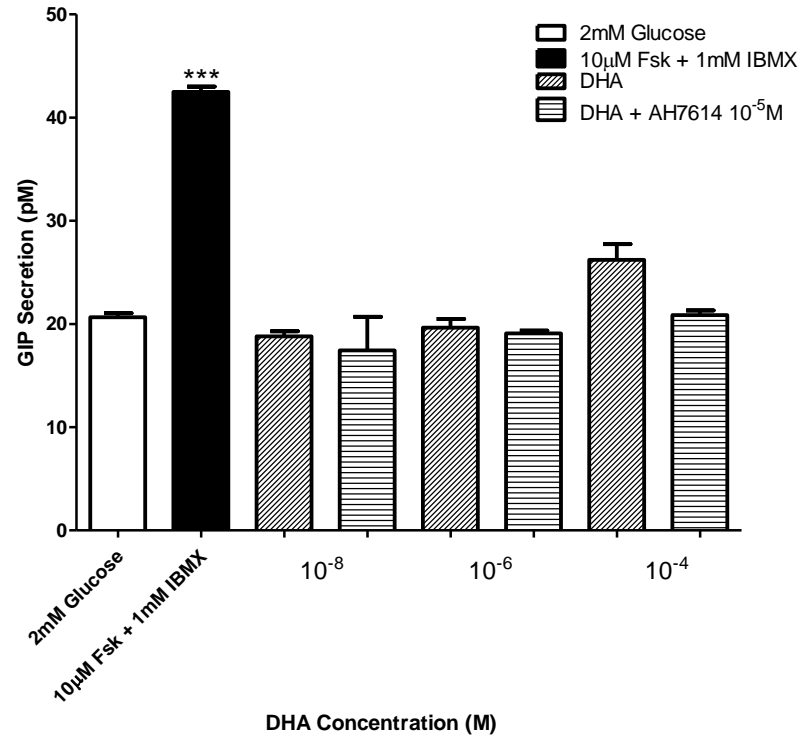
Figure 5.4: Effects of endogenous GPR120 agonist ALA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



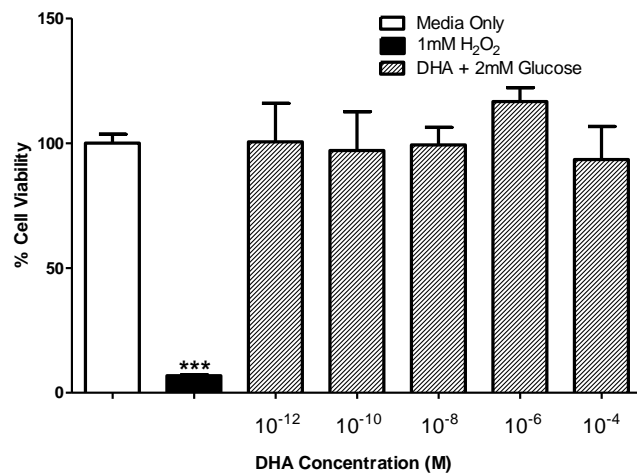
Effect of GPR120 agonist ALA (10^{-8} - 10^{-4} M) and antagonist AH7614 on GIP secretion (A) and cell viability (10^{-12} - 10^{-4} M) (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 5.5: Effects of endogenous GPR120 agonist DHA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



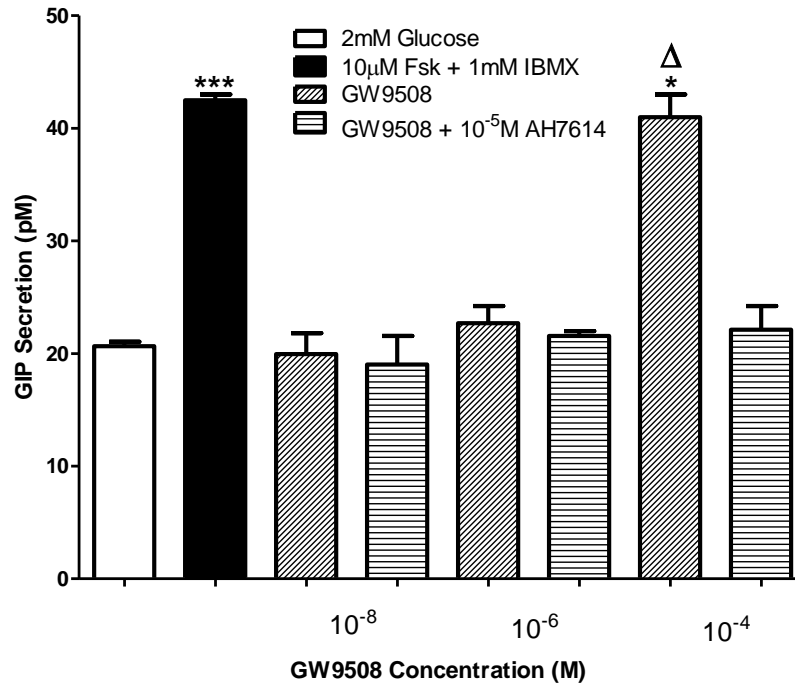
B.



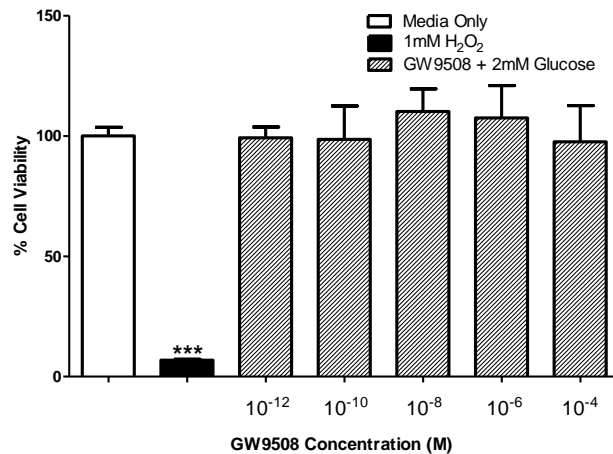
Effect of GPR120 agonist DHA (10^{-8} - 10^{-4} M) and antagonist AH7614 on GIP secretion (A) and cell viability (10^{-12} - 10^{-4} M) (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 5.6: Effects of synthetic GPR120 agonist GW9508 on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



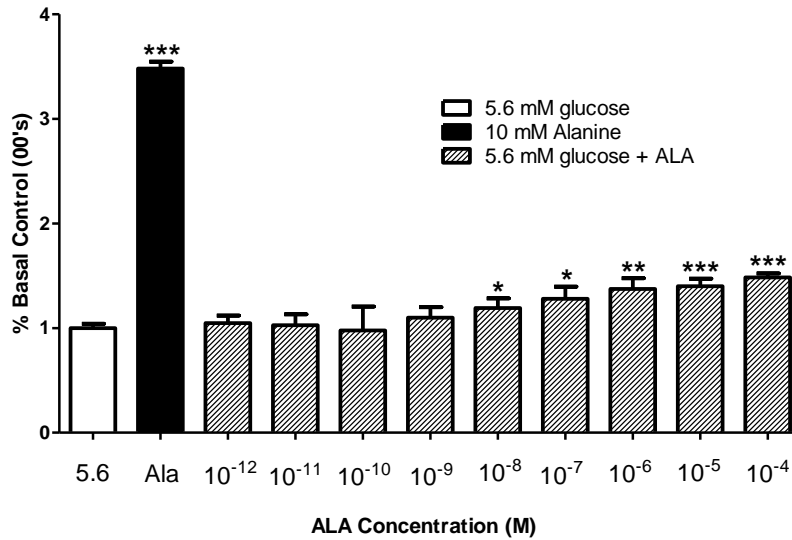
B.



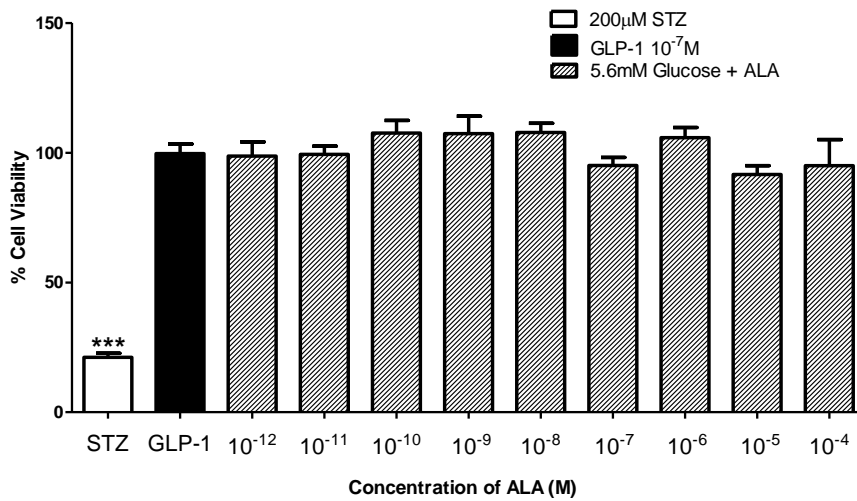
Effect of GPR120 agonist GW9508 (10^{-8} - 10^{-4} M) and antagonist AH7614 on GIP secretion (A) and cell viability (10^{-12} - 10^{-4} M) (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability. Δ $p < 0.05$ compared to treatment with antagonist in the presence of agonist at the same concentration.

Figure 5.7: Effects of endogenous GPR120 agonist ALA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



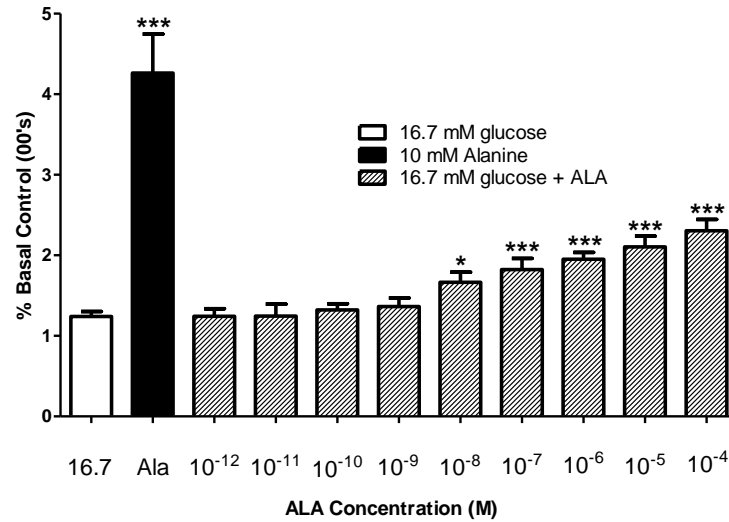
B.



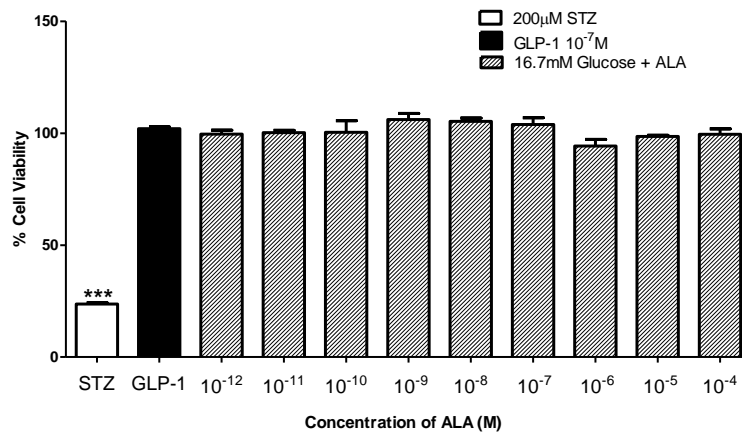
Effect of ALA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 5.8: Effects of endogenous GPR120 agonist ALA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.



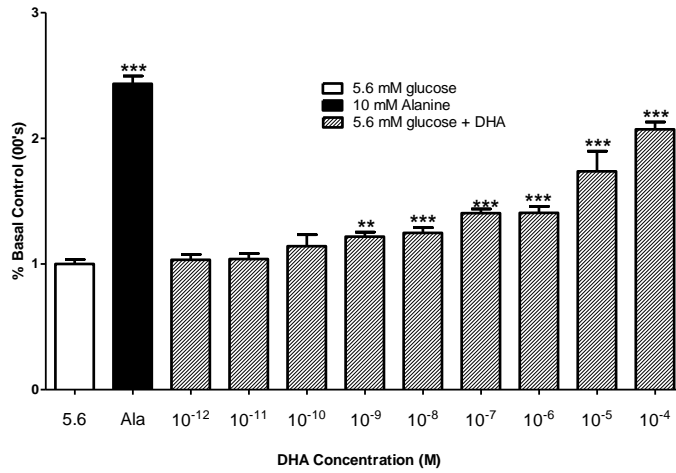
B.



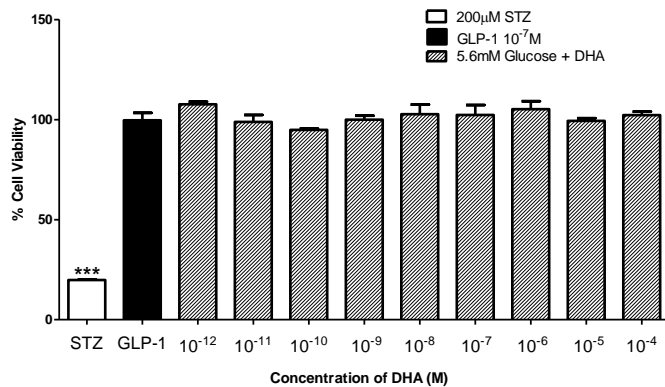
Effect of ALA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 5.9: Effects of endogenous GPR120 agonist DHA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



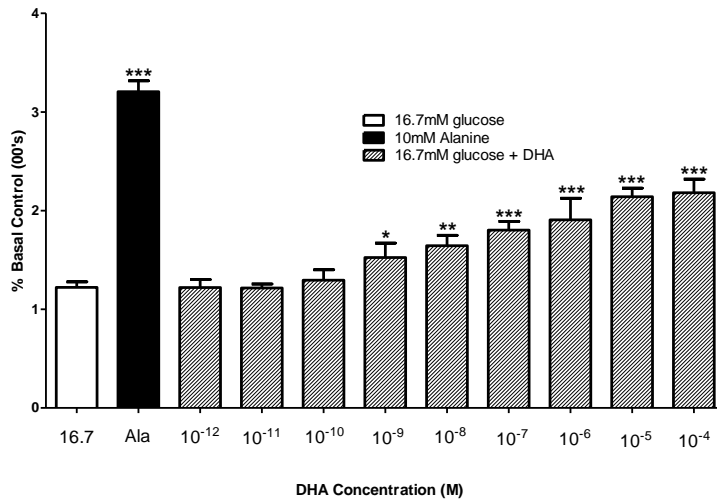
B.



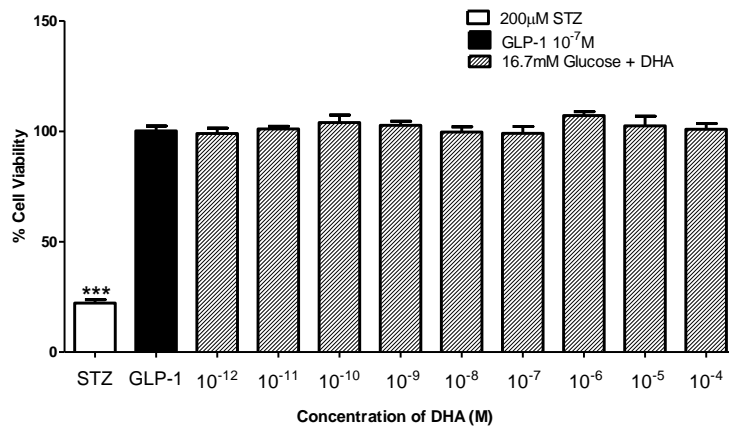
Effect of DHA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 5.10: Effects of endogenous agonist DHA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.



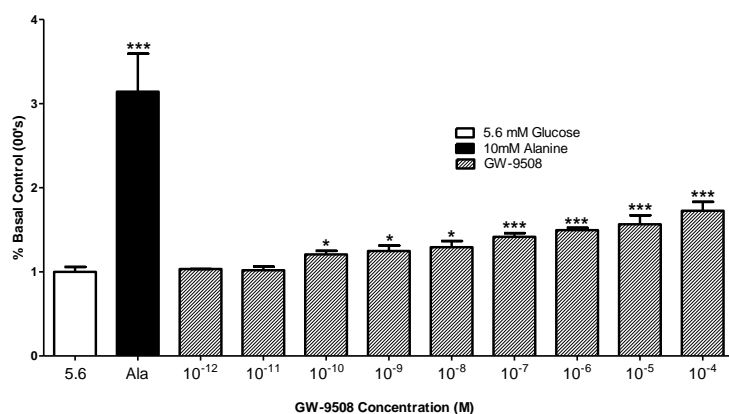
B.



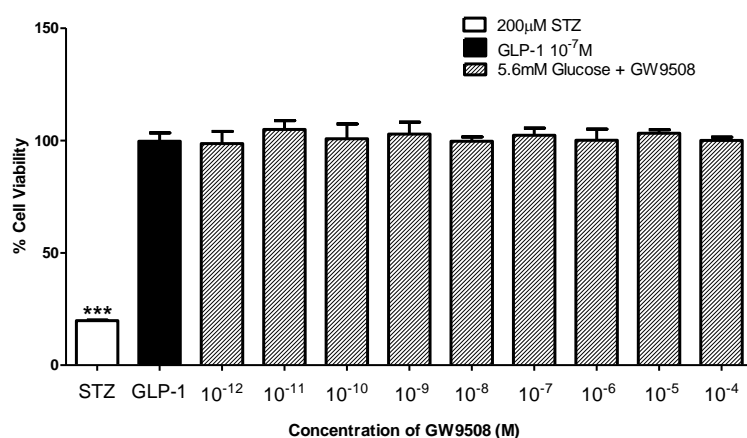
Effect of DHA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 5.11: Effects of synthetic GPR120 agonist GW9508 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



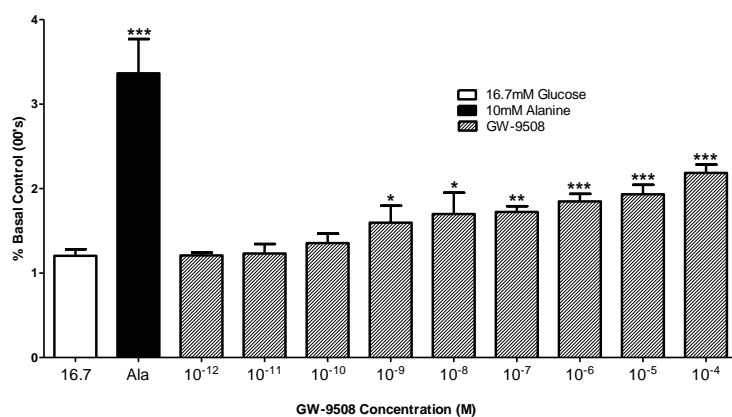
B.



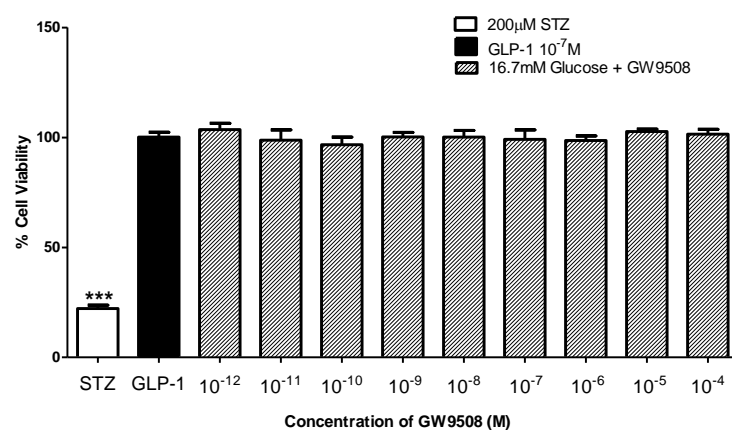
Effect of GW9508 (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 5.12: Effects of synthetic GPR120 agonist GW9508 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.



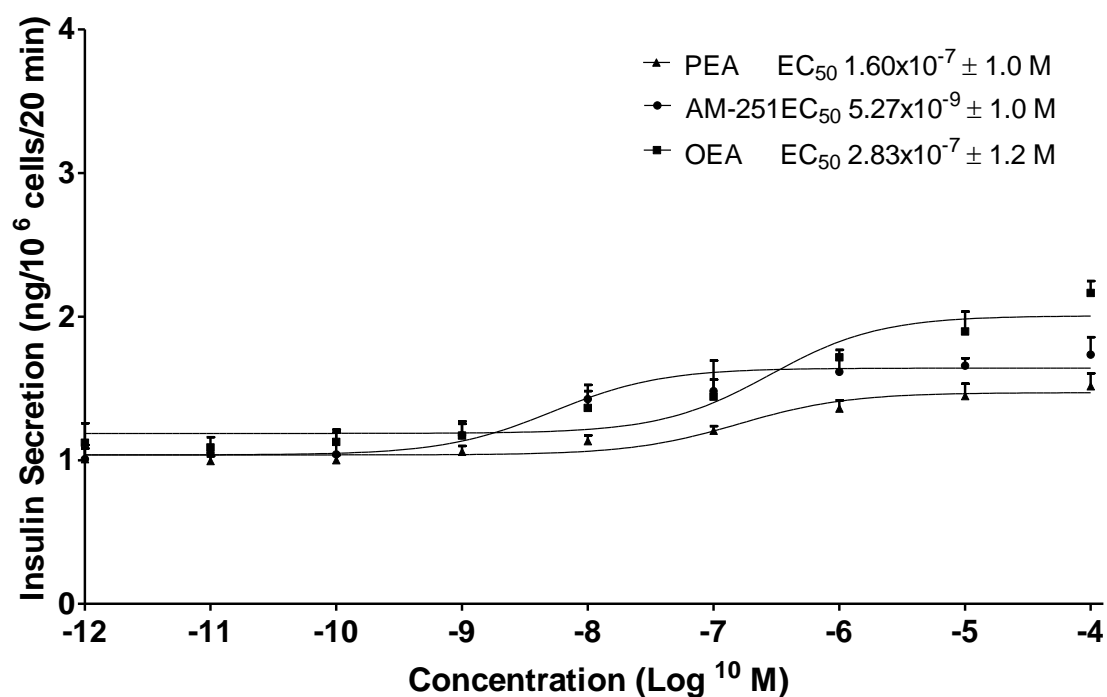
B.



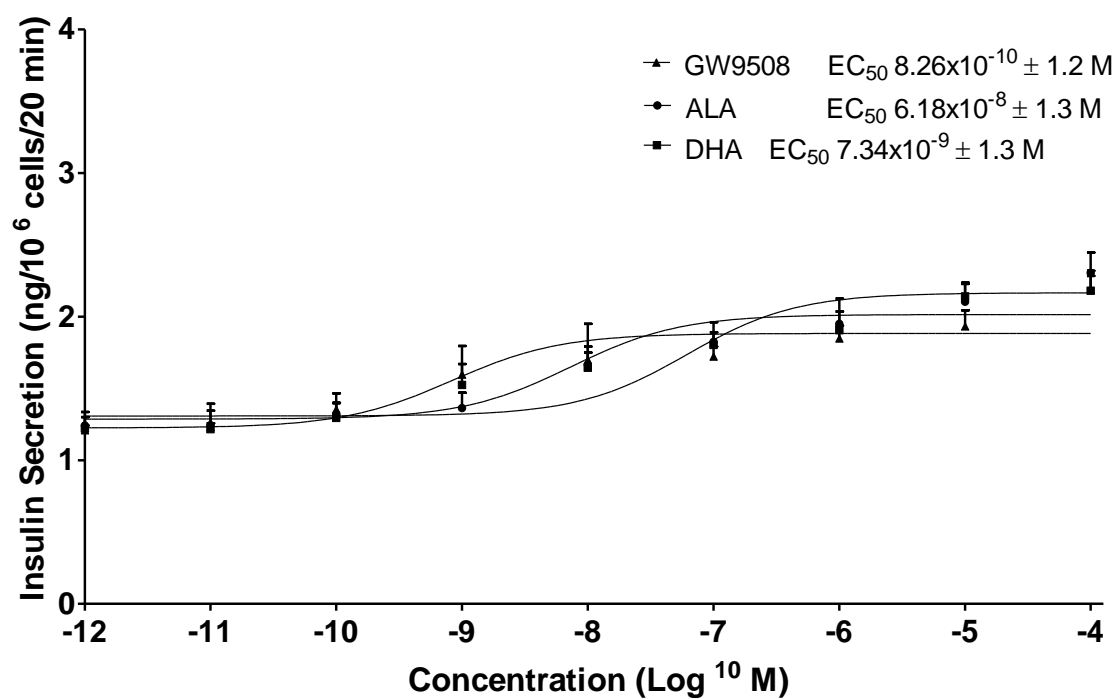
Effect of GW9508 (10⁻¹²-10⁻⁴M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * p<0.05, ** p<0.01 and *** p<0.001 compared to basal glucose control for insulin secretion and GLP-1 (10⁻⁷M) non-toxic control for cell viability.

Figure 5.13: EC₅₀ of GPR120 agonists ALA, DHA and GW9508 in BRIN-BD11 cells

A.

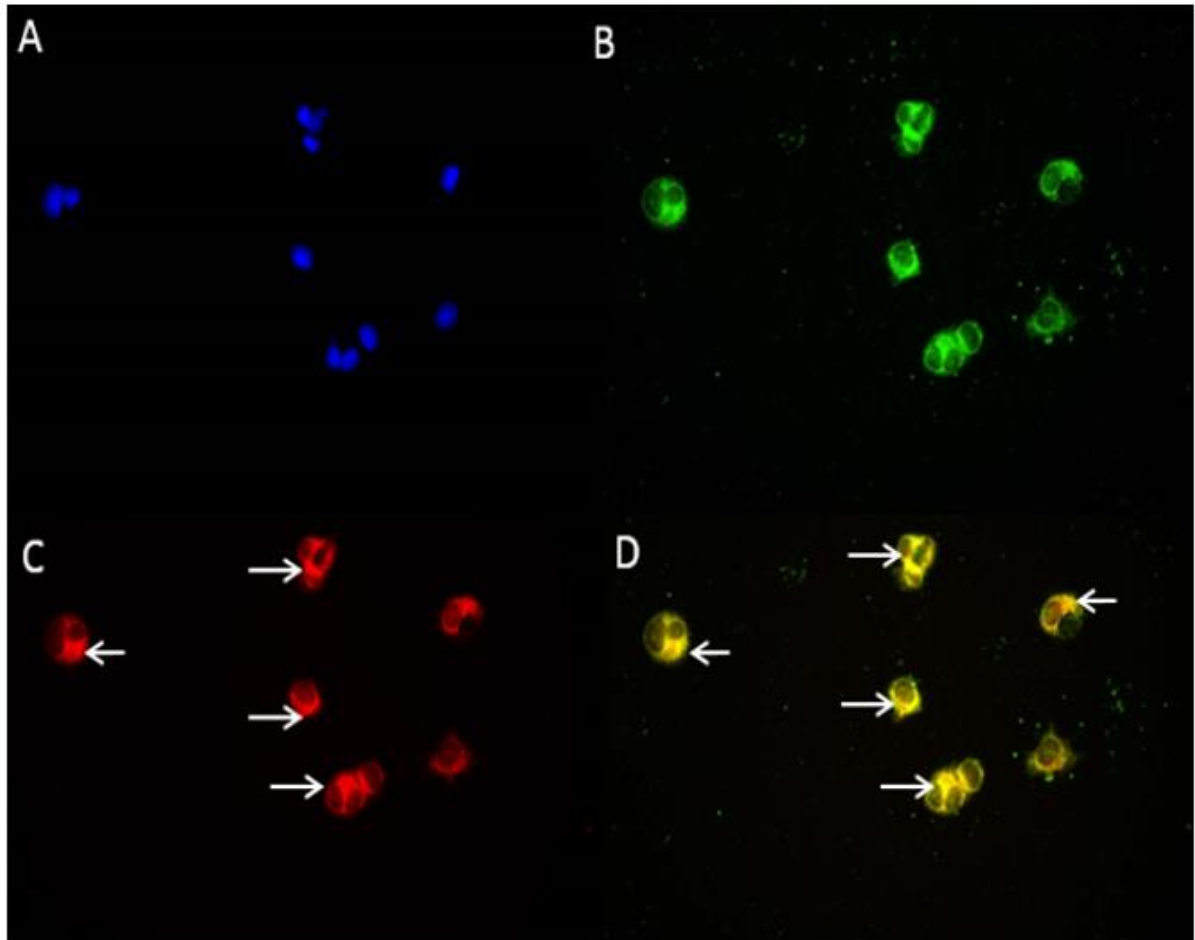


B.



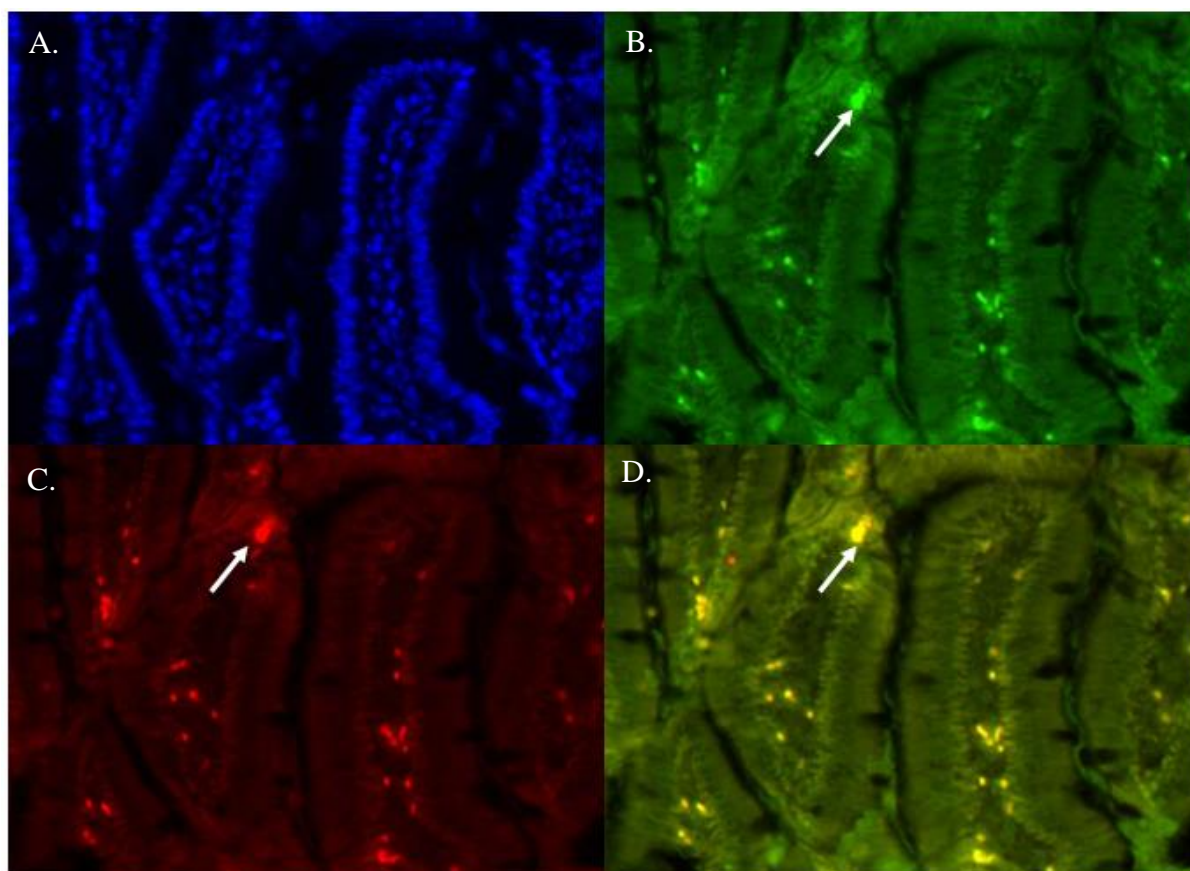
EC₅₀ values for GPR120 agonists ALA, DHA and GW9508 in BRIN-BD11 cells in 5.6mM glucose (A) and 16.7mM glucose (B).

Figure 5.14: Double immunofluorescence staining of GLP-1 and GPR120 in intestinal GLUTag cells



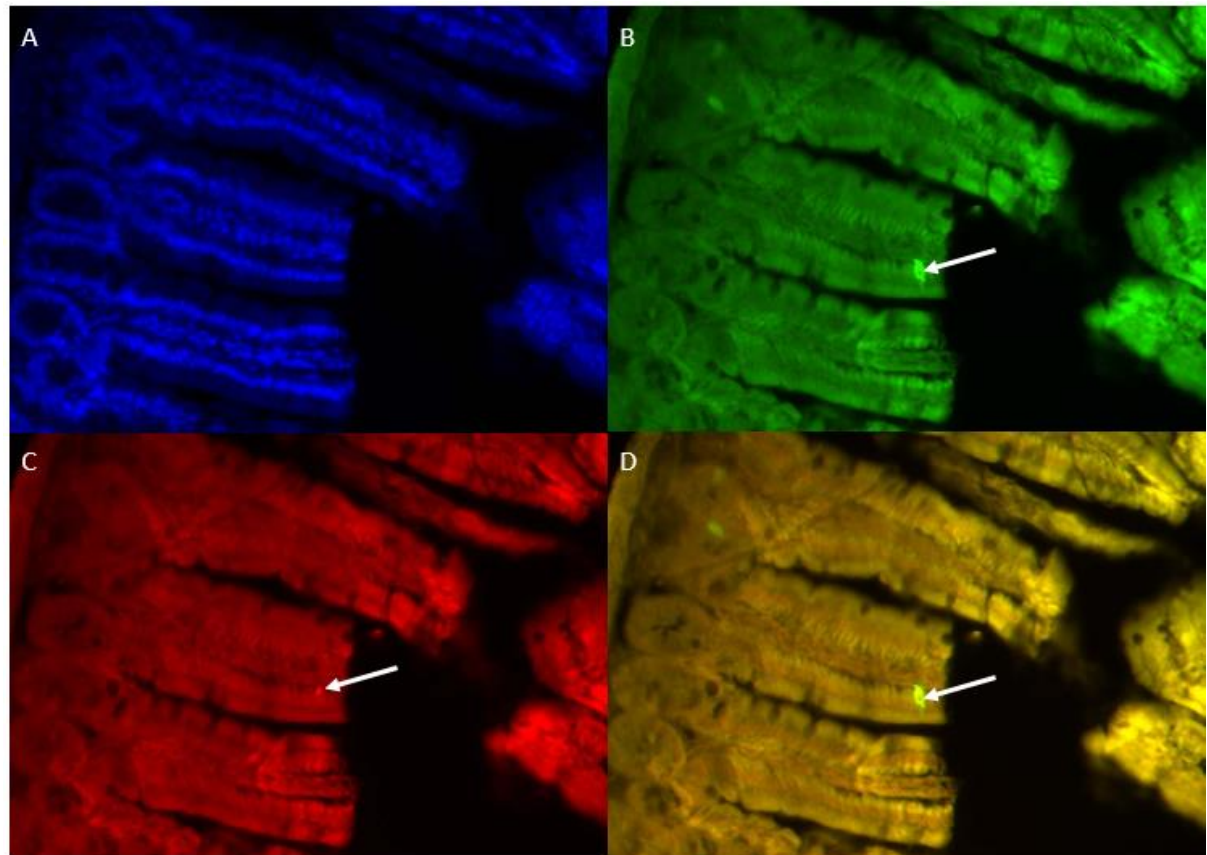
Distribution of (A) DAPI, (B) GLP-1, (C) GPR120 and (D) combined fluorescence of GLP-1 and GPR120 in GLUTag cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.15: Double immunofluorescence staining of GLP-1 and GPR120 in lean NIH Swiss mouse small intestine



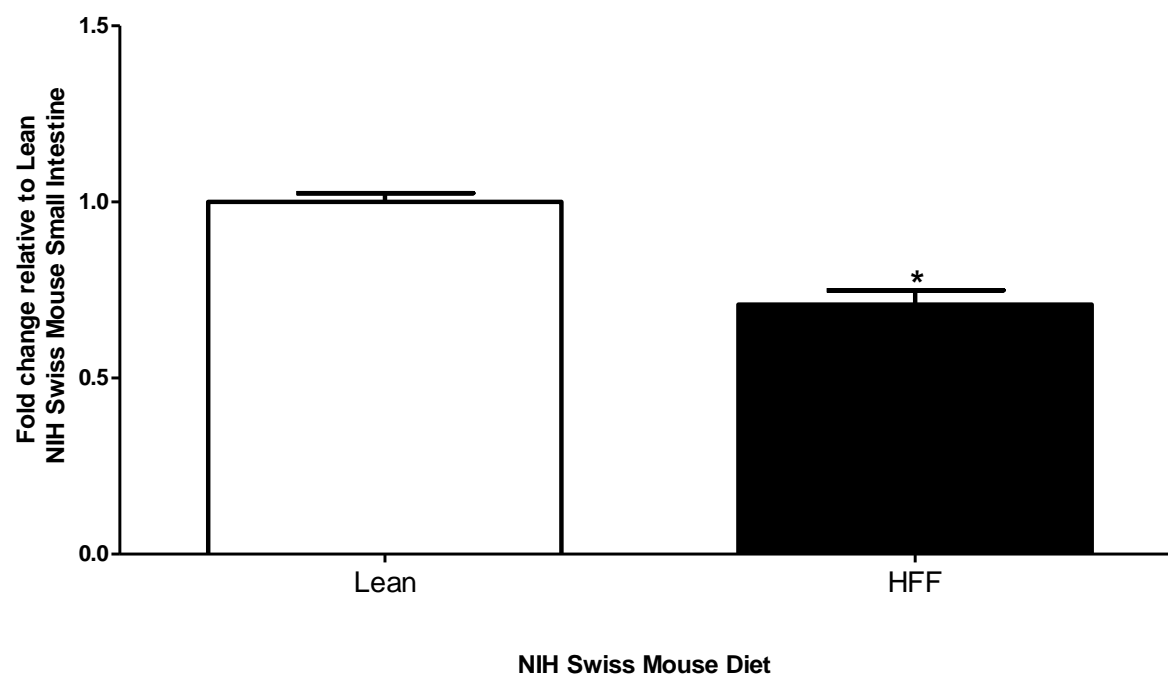
Distribution of (A) DAPI, (B) GLP-1, (C) GPR120 and (D) combined fluorescence of GLP-1 and GPR120 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.16: Double immunofluorescence staining of GLP-1 and GPR120 in HFF NIH Swiss mouse small intestine



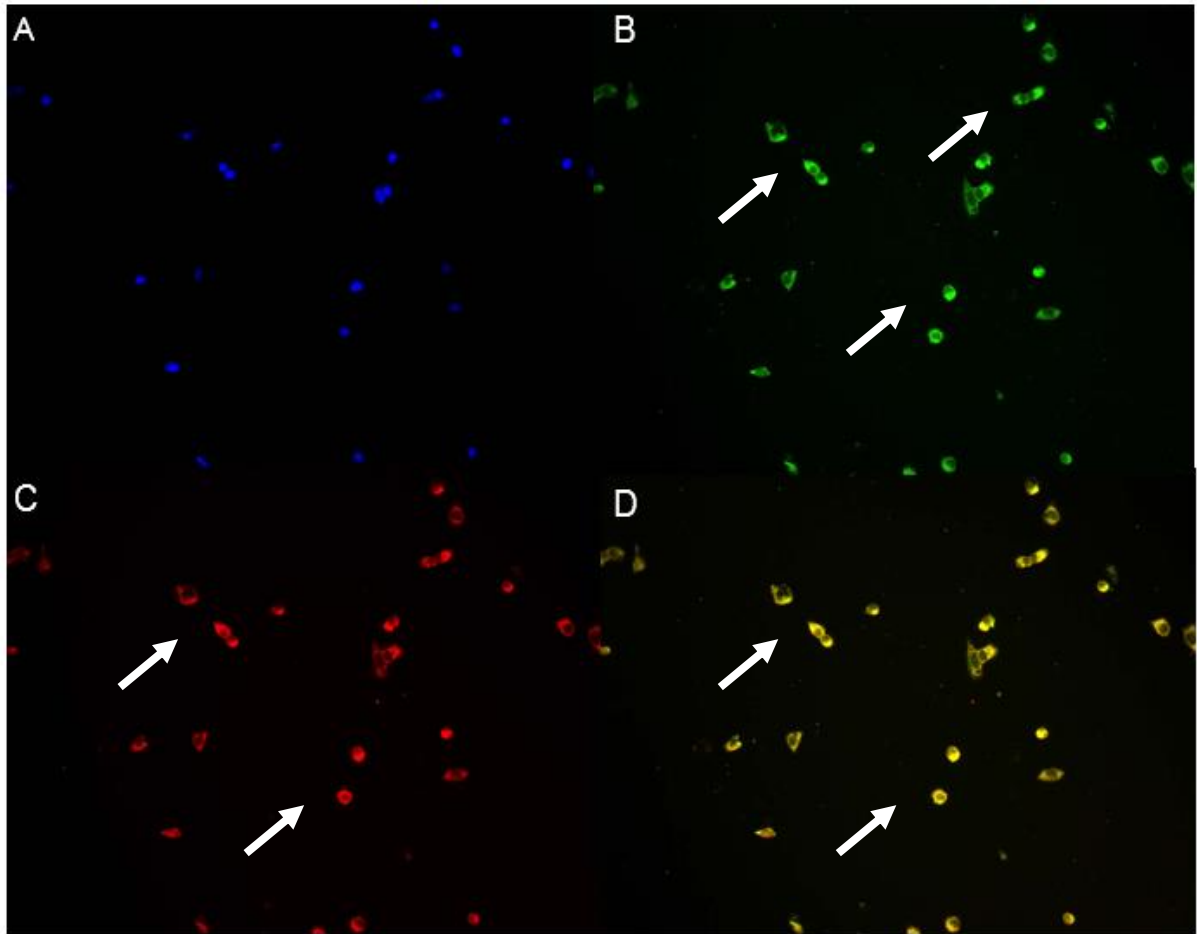
Distribution of (A) DAPI, (B) GLP-1, (C) GPR120 and (D) combined fluorescence of GLP-1 and GPR120 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.17: GPR120 gene expression in lean and HFF NIH Swiss mouse small intestine



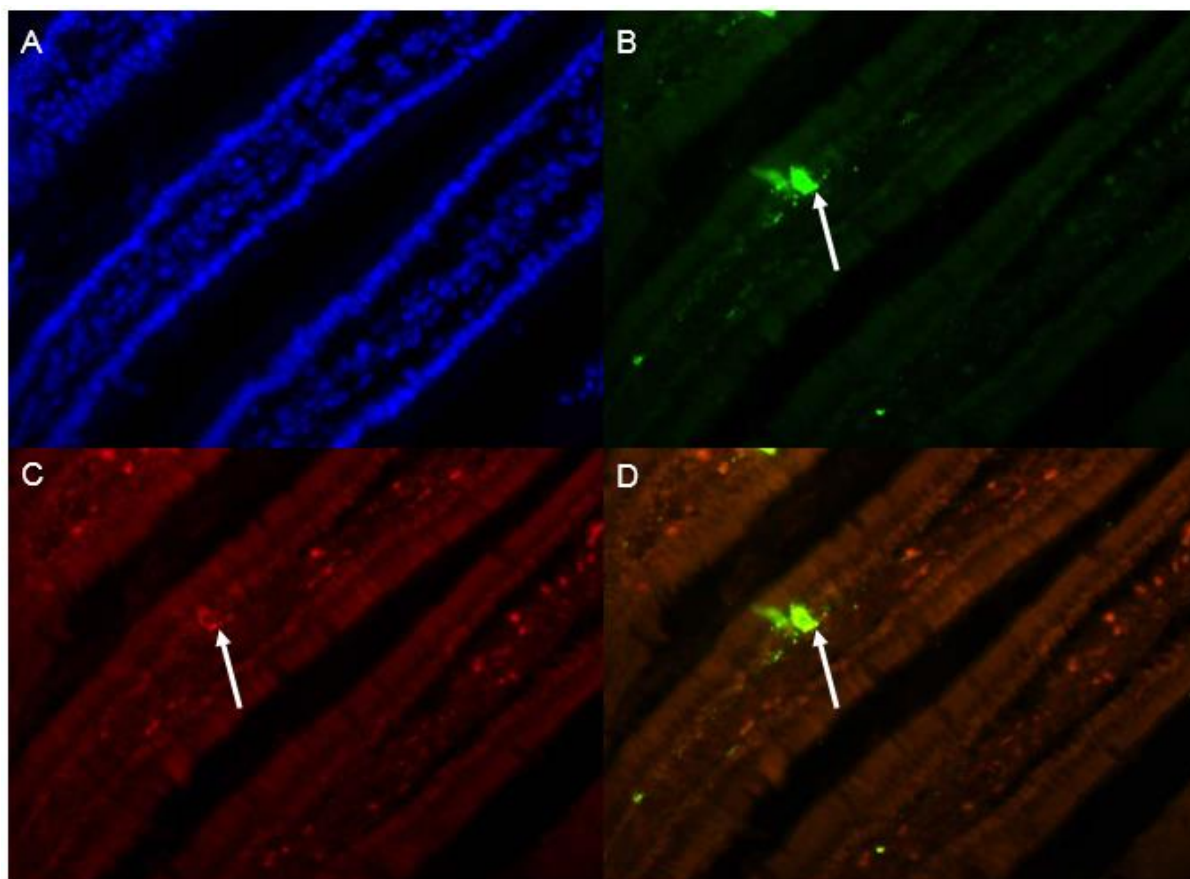
Effect of HFF diet on GPR120 gene expression in male NIH Swiss mouse small intestine. Results are \pm SEM (n=6). * $p < 0.05$ compared to lean mice.

Figure 5.18: Double immunofluorescence staining of GIP and GPR120 in pGIPneo STC-1 cells



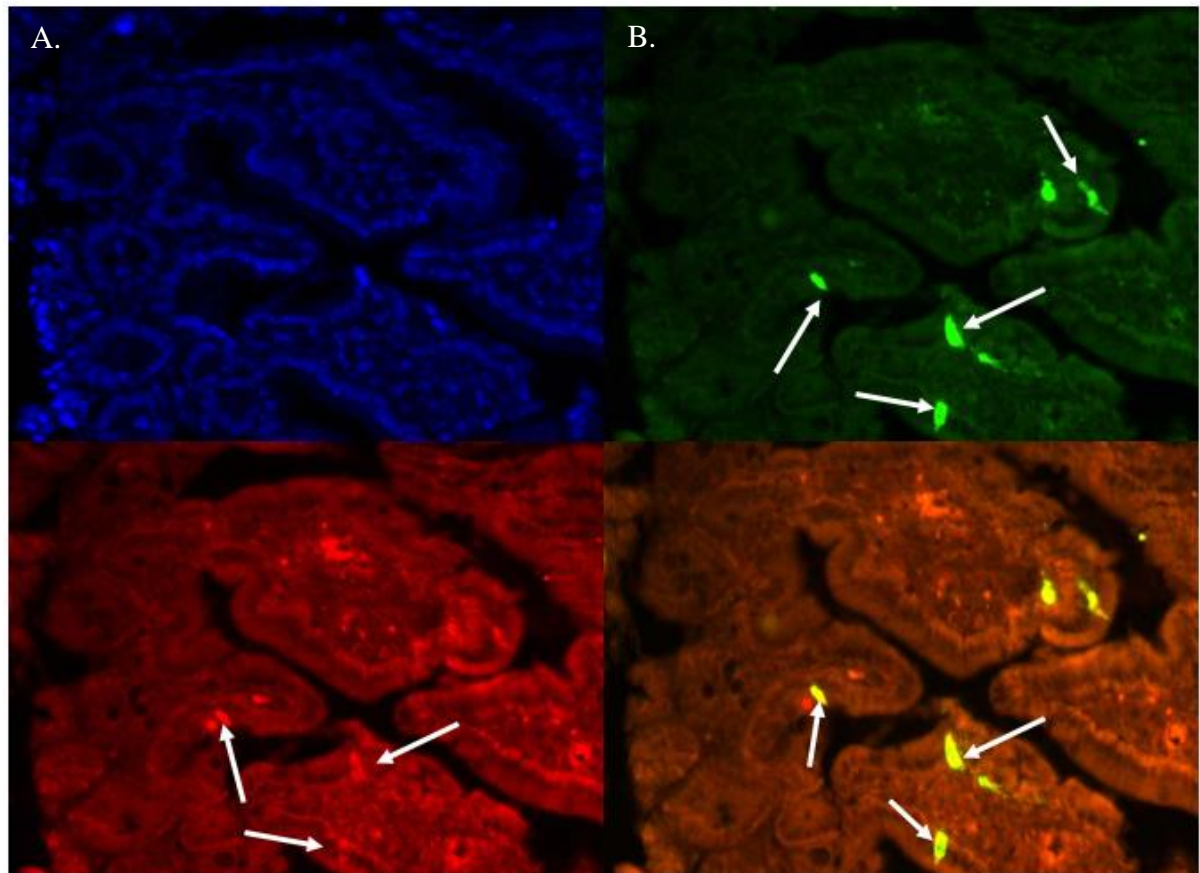
Distribution of (A) DAPI, (B) GIP, (C) GPR120 and (D) combined fluorescence of GIP and GPR120 in pGIPneo STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.19: Double immunofluorescence staining of GIP and GPR120 in lean NIH Swiss mouse small intestine



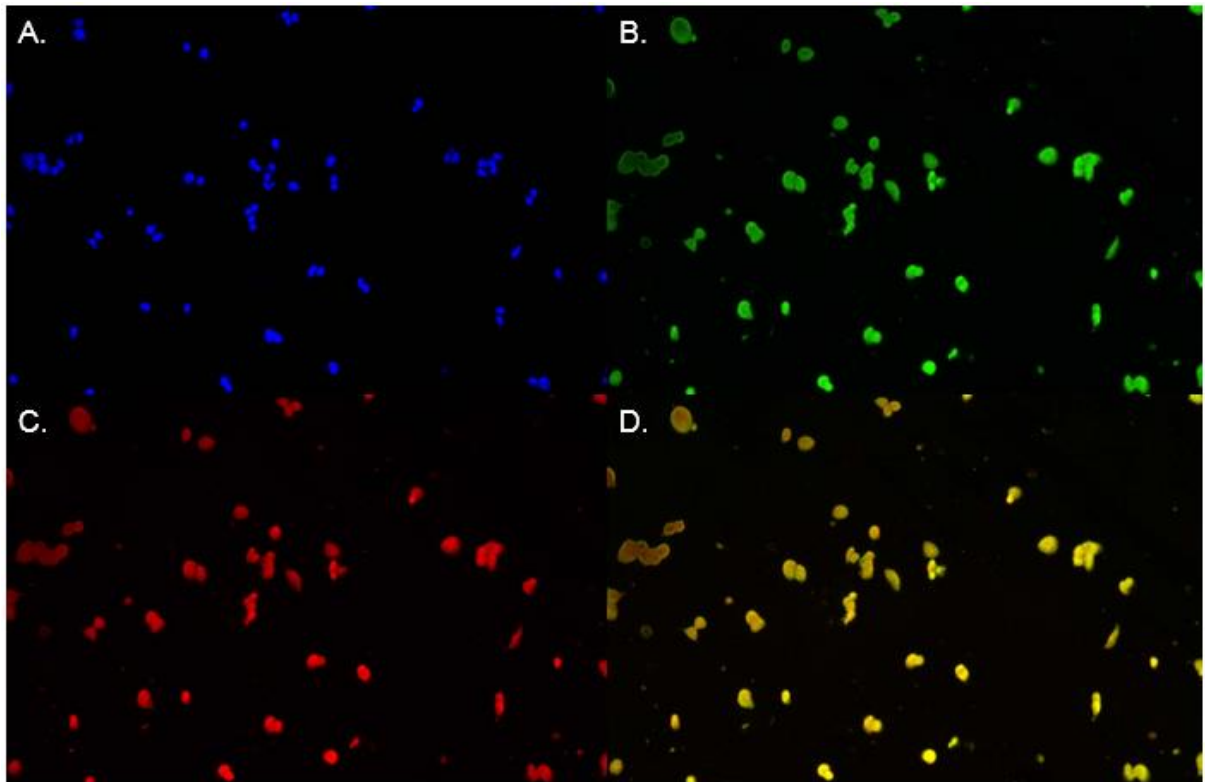
Distribution of (A) DAPI, (B) GIP, (C) GPR120 and (D) combined fluorescence of GIP and GPR120 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.20: Double immunofluorescence staining of GIP and GPR120 in HFF NIH Swiss mouse small intestine



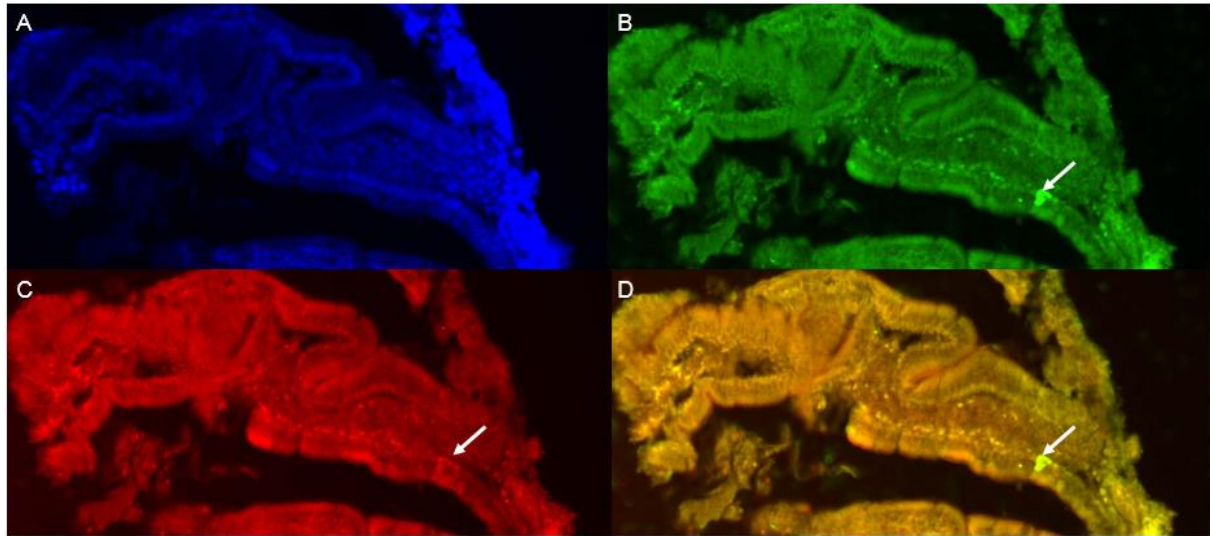
Distribution of (A) DAPI, (B) GIP, (C) GPR120 and (D) combined fluorescence of GIP and GPR120 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.21: Double immunofluorescence staining of PYY and GPR120 in STC-1 cells



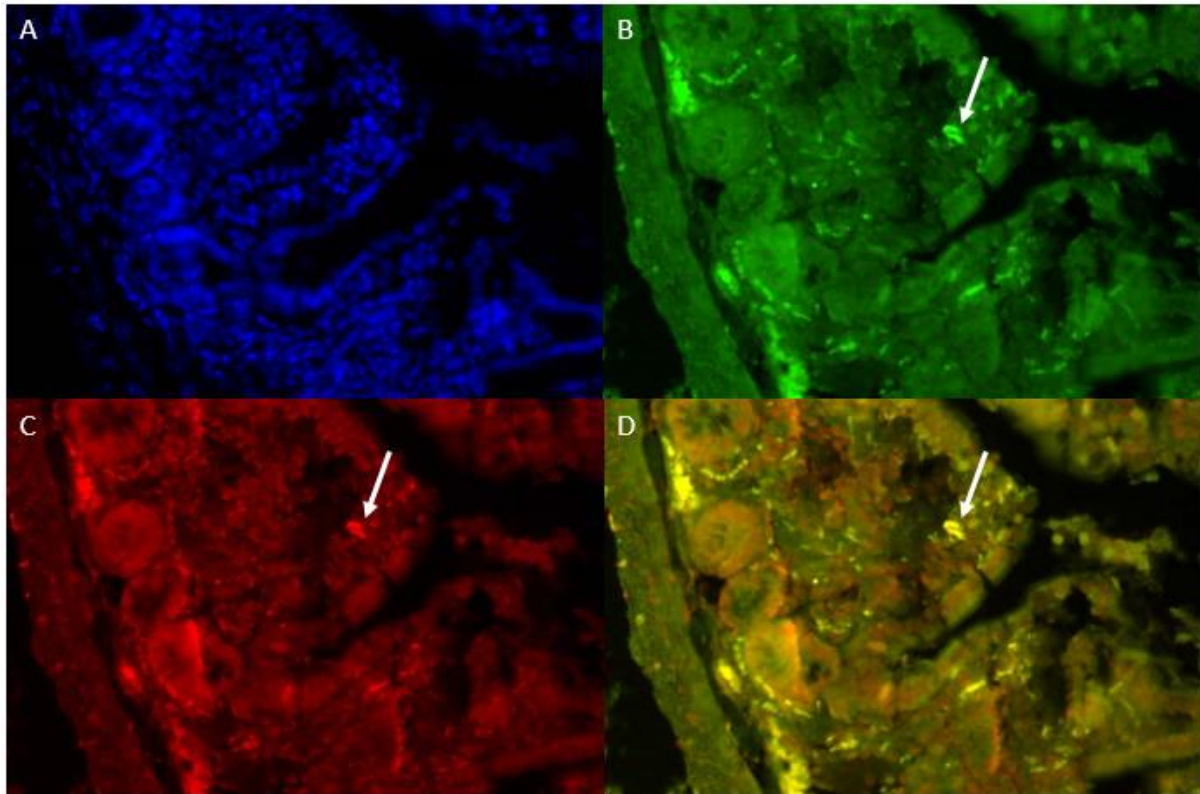
Distribution of (A) DAPI, (B) PYY, (C) GPR120 and (D) combined fluorescence of PYY and GPR120 in STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.22: Double immunofluorescence staining of PYY and GPR120 in lean NIH Swiss mouse small intestine



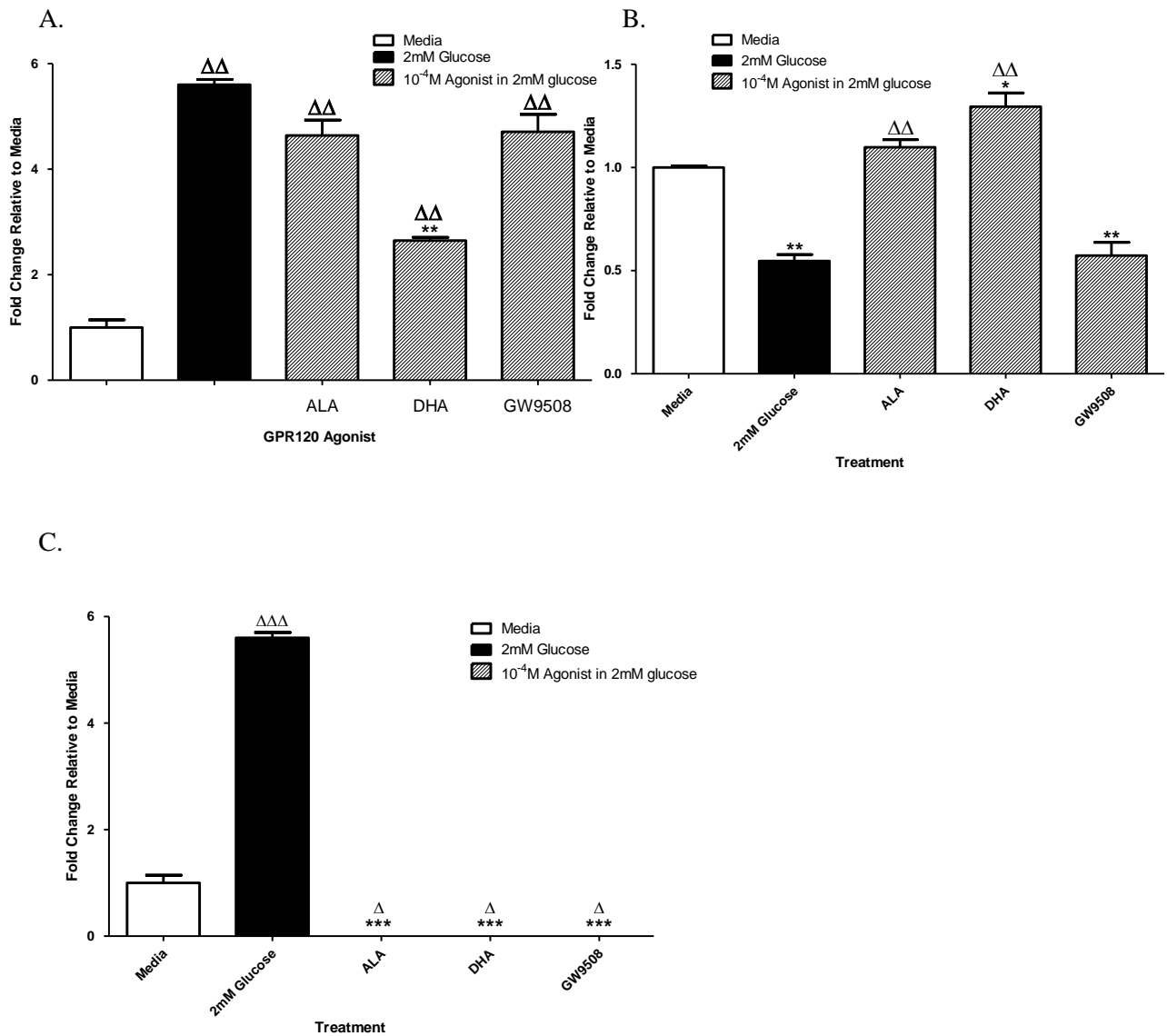
Distribution of (A) DAPI, (B) PYY, (C) GPR120 and (D) combined fluorescence of PYY and GPR120 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 5.23: Double immunofluorescence staining of PYY and GPR120 in HFF NIH Swiss mouse small intestine



Distribution of (A) DAPI, (B) PYY, (C) GPR120 and (D) combined fluorescence of PYY and GPR120 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

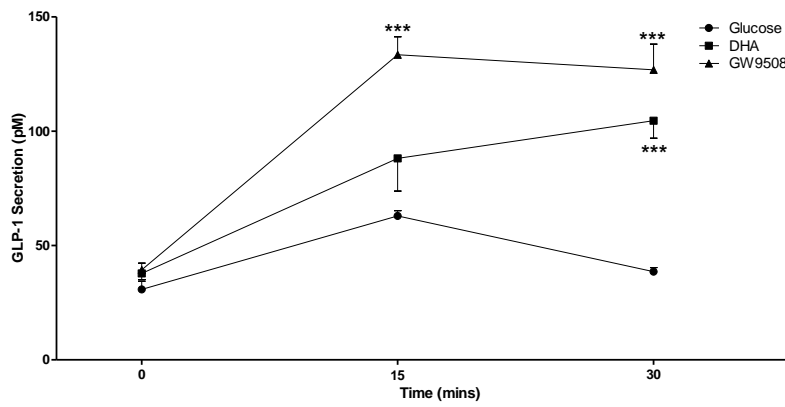
Figure 5.24: Effect of GPR120 Agonists on GPR120, proglucagon and PC1 gene expression in GLUTag cells



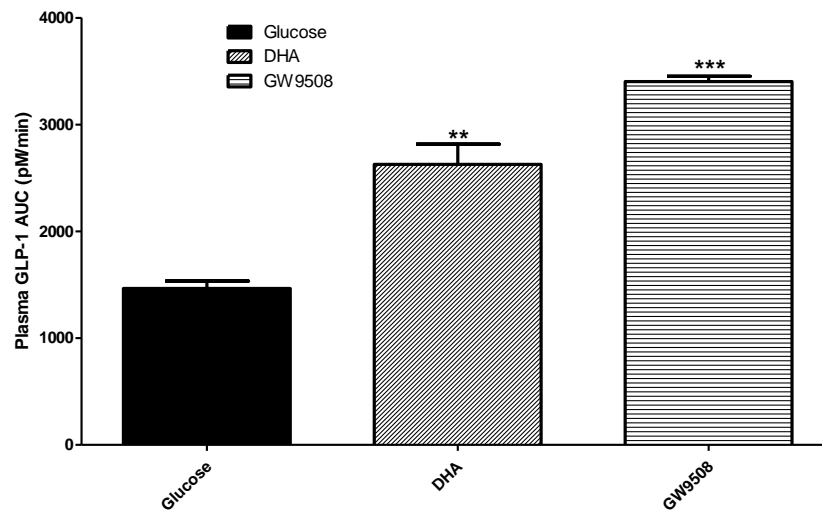
Effects of GPR120 agonists on GPR120 (A) proglucagon (B) and PC1 (C) gene expression in intestinal GLUTag cells at 2mM glucose. Results are \pm SEM (n=3). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle control. Δ $p < 0.05$ and $\Delta\Delta$ $p < 0.01$ compared to untreated control.

Figure 5.25: Effects of GPR120 agonists on plasma GLP-1 in male Swiss TO mice

A.



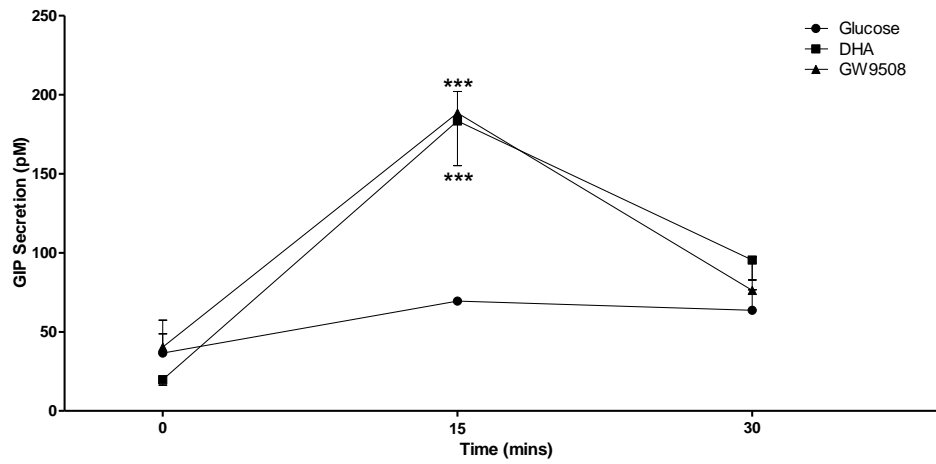
B.



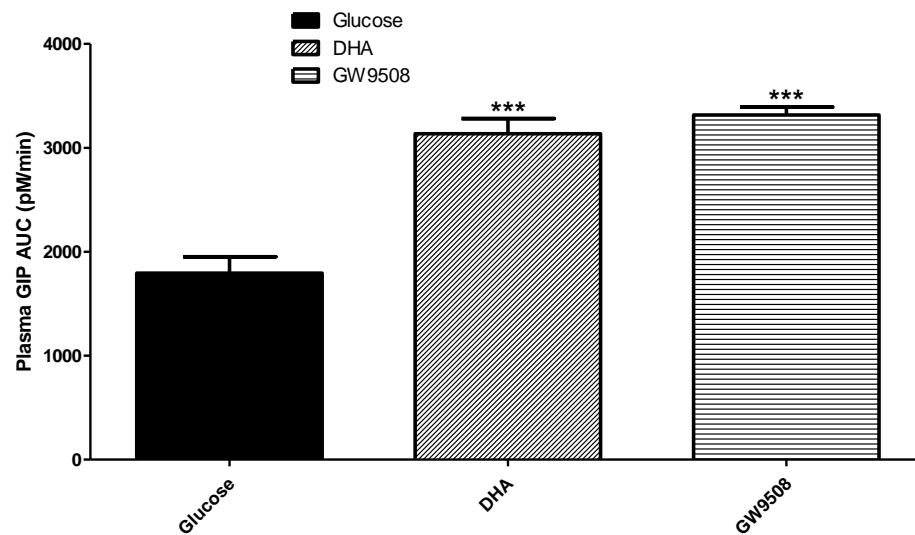
Glucose (18mmol/kg BW) or glucose in combination with a GPR120 agonist (0.1 μ mol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GLP-1 of mice treated with DHA and GW9508 (B) AUC of A. ** p<0.01 and *** p<0.001 compared to glucose alone.

Figure 5.26: Effects of GPR120 agonists on plasma GIP in male Swiss TO mice

A.



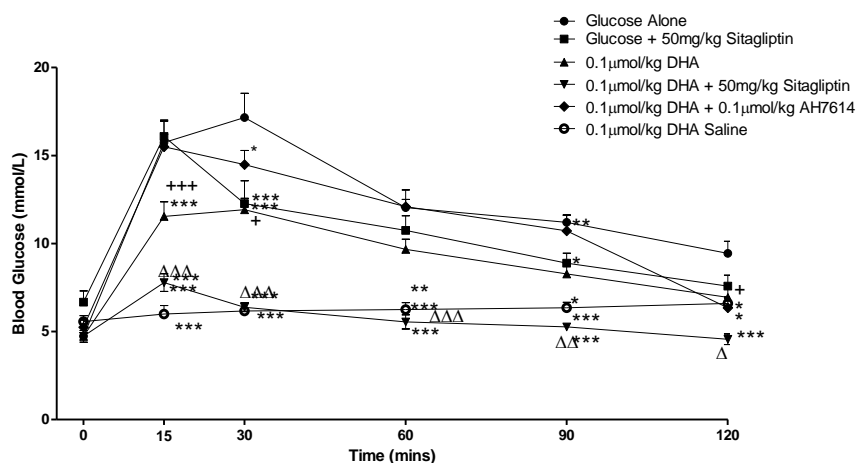
B.



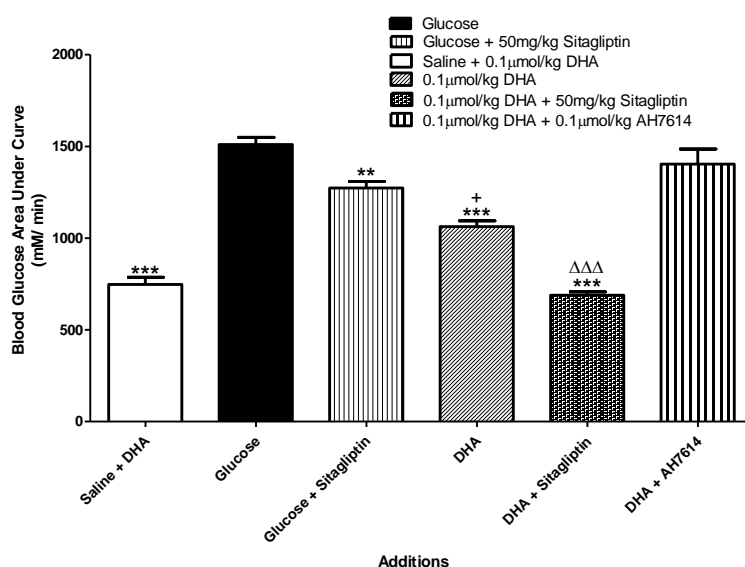
Glucose (18mmol/kg BW) or glucose in combination with a GPR120 agonist (0.1 μ mol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GIP of mice treated with DHA and GW9508 (B) AUC of A. *** $p < 0.001$ compared to glucose alone.

Figure 5.27: Acute effects of oral administration of GPR120 agonist DHA on blood glucose in male Swiss TO mice

A.



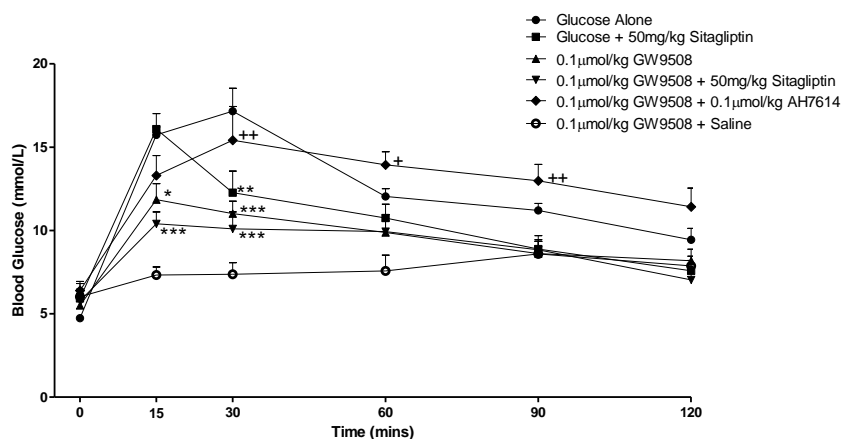
B.



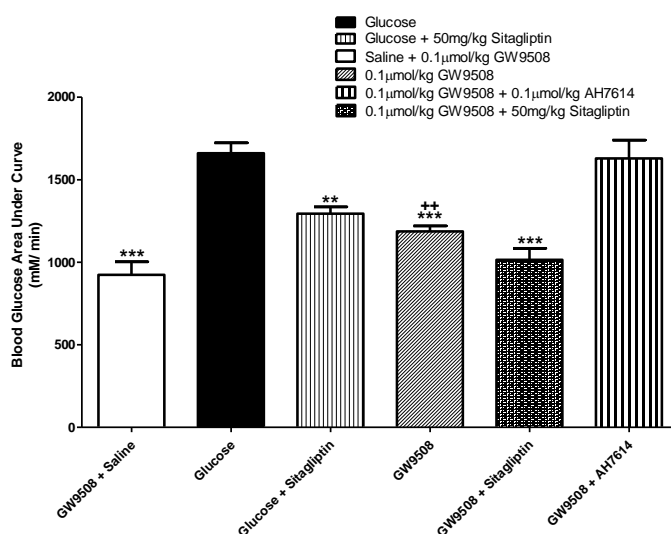
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with DHA (0.1µmol/kg BW), glucose in combination with DHA and the GPR120 antagonist AH7614 (0.1µmol/kg BW), glucose in combination with DHA and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with a DHA were all administered orally to male Swiss TO mice (n=6). (A) Blood glucose of mice treated with DHA (B) AUC of A. * p<0.05, ** p<0.01 and *** p<0.001 compared to glucose alone. + p<0.05 and +++ p<0.001 compared to agonist in combination with antagonist. ΔΔΔ p<0.001 compared to GPR120 agonist treatment.

Figure 5.28: Acute effects of oral administration of GPR120 agonist GW9508 on blood glucose in male Swiss TO mice

A.



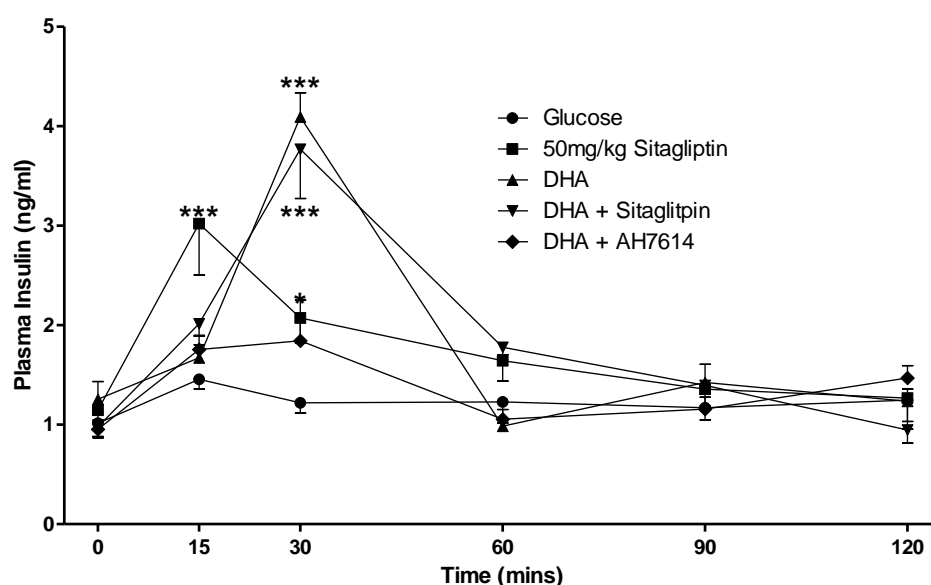
B.



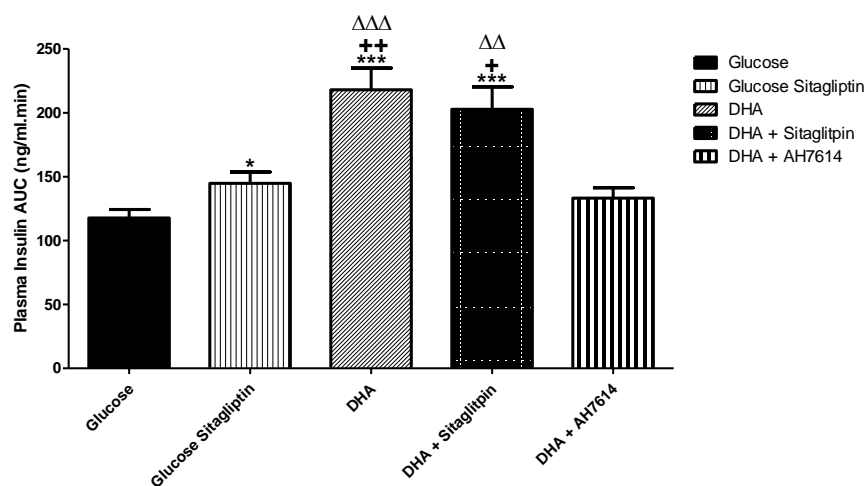
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with GW9508 (0.1µmol/kg BW), glucose in combination with GW9508 and the GPR120 antagonist AH7614 (0.1µmol/kg BW), glucose in combination with GW9508 and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with GW9508 were all administered orally to male Swiss TO mice. (A) Blood glucose of mice treated with GW9508 (B) AUC of A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose alone. + $p < 0.05$ and ++ $p < 0.01$ compared to agonist in combination with antagonist.

Figure 5.29: Effects of GPR120 agonist DHA on plasma insulin in male Swiss TO mice

A.



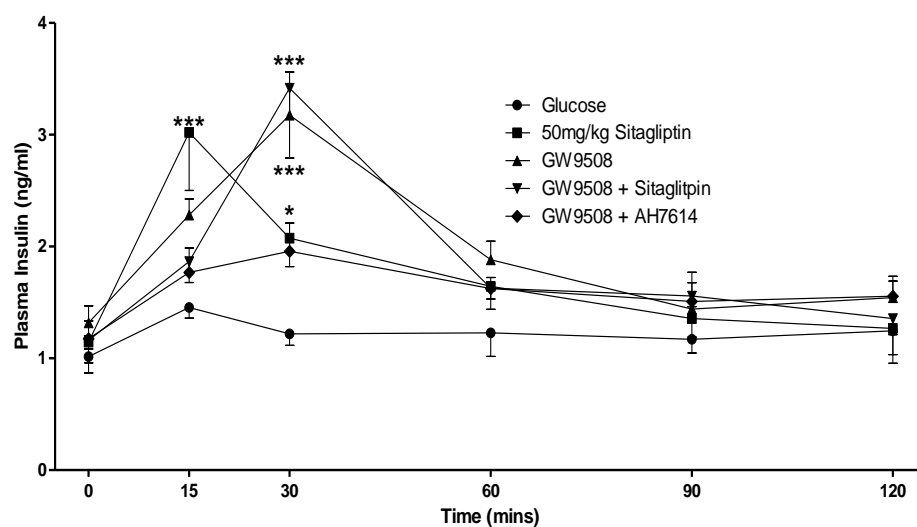
B.



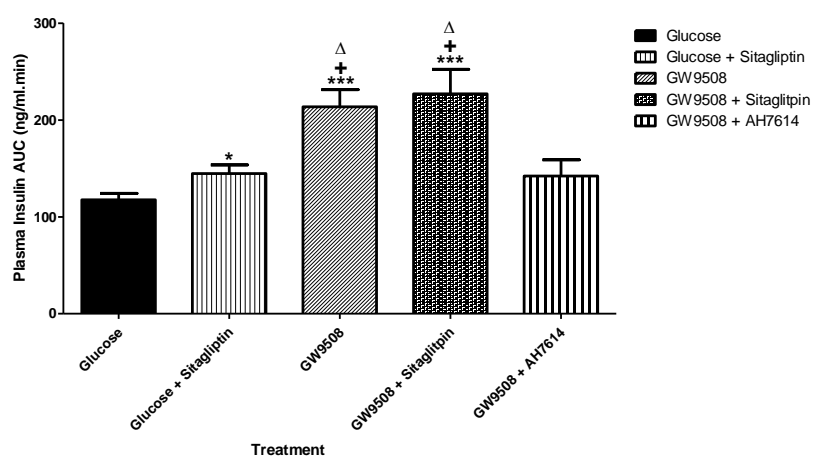
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with DHA (0.1μmol/kg BW), glucose in combination with DHA and the GPR120 antagonist AH7614 (0.1μmol/kg BW), glucose in combination with DHA and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with DHA were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with DHA (B) AUC of A. * p<0.05 and *** p<0.001 compared to glucose treatment alone. + p<0.05 and ++ p<0.01 compared to agonist in combination with antagonist. ΔΔ P<0.01 and ΔΔΔ p<0.001 compared to glucose and Sitagliptin.

Figure 5.30: Effects of GPR120 agonist GW9508 on plasma insulin in male Swiss TO mice

A.



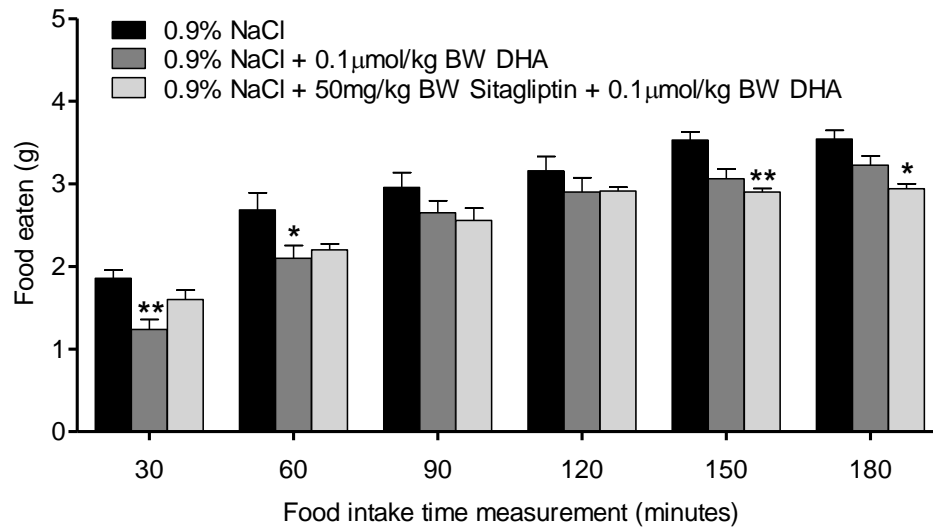
D.



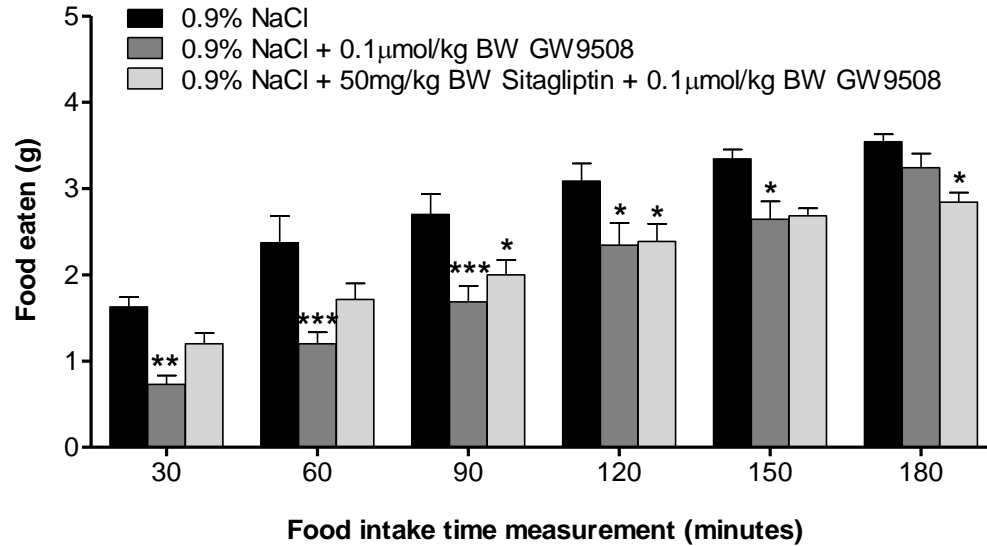
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with GW9508 (0.1μmol/kg BW), glucose in combination with GW9508 and the GPR120 antagonist AH7614 (0.1μmol/kg BW), glucose in combination with GW9508 and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with GW9508 were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with GW9508 (B) AUC of A. * p<0.05, ** p<0.01 and *** p<0.001 compared to glucose treatment alone. + p<0.05 compared to agonist treatment in combination with the GPR120 antagonist AH7614. Δ p<0.05 compared to glucose and Sitagliptin.

Figure 5.31: Effects of GPR120 agonists on feeding in trained male Swiss TO mice

A.



B.



Saline (0.9%) or saline and a GPR120 agonist (0.1 μmol/kg BW) were administered orally to Swiss TO mice which had been trained to eat for 3 hrs daily (n=8). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to saline.

Chapter 6

Investigating the acute and long term
effects of GPR55 activation on incretin
hormone secretion and glucose
homeostasis

6.1: Overview of results

Novel cannabinoid receptor GPR55 is expressed throughout the body and may regulate a variety of physiological processes. Activation of GPR55 by fatty acids has been shown to have an insulintropic effect both *in vitro* and *in vivo* as well as after acute and long term administration. Recently research has been published demonstrating the effect of GPR55 activation by LPI on GLP-1 secretion. The effects of GPR55 activation on GIP and PYY have yet to be elucidated, however administration of Abn-CBD in GIP receptor knockout mice has shown that GPR55 agonists may play a role in GIP secretion. The current study investigates the effects of GPR55 agonists on intestinal hormone secretion in various cell lines *in vitro* while also examining these effects *in vivo*. In order to confirm that GPR55 agonists are non-toxic, cytotoxicity was determined by Alamar Blue and MTT assay. The expression of GPR55 *in vitro* and *ex vivo* was determined using immunofluorescence and qPCR. The *in vivo* effects of Abn-CBD and AM251 (0.1µM/kg BW) were determined in fasted male Swiss TO mice (n=6) both alone and each in combination with Sitagliptin (50mg/kg BW). The biological effects of chronic treatment of GPR55 agonist Abn-CBD both as a monotherapy and a combination therapy with Sitagliptin were examined in streptozotocin induced diabetic Swiss TO mice (n=6).

Treatment of GLUTag cells with GPR55 agonists in 2mM glucose increased GLP-1 secretion *in vitro*. Abn-CBD increased GLP-1 secretion 1.7 to 2.4-fold (10^{-8} - 10^{-4} M, $p<0.05$) and this was decreased 1.4 to 1.7-fold (10^{-8} - 10^{-4} M, $p<0.05$). AM251, increased GLP-1 secretion 1.4 to 1.7-fold (10^{-8} M to 10^{-6} M, $p<0.01$ - $p<0.001$) compared to 2mM glucose and this was reduced 1.7 to 2.1-fold (10^{-10} to 10^{-6} M, $p<0.01$) in the presence of 10^{-5} M CBD. PEA increased GLP-1 secretion from GLUTag cells 1.3 to 2.5-fold (10^{-8} M to 10^{-4} M, $p<0.01$ to $p<0.001$) compared to basal control with the addition of 10^{-5} M CBD reducing the effect of PEA agonism by 1.6, 1.4 and 1.9-fold (10^{-8} to 10^{-4} M, $p<0.05$ to $p<0.001$). OEA (10^{-6} to 10^{-4} M) promoted GLP-1 secretion 1.8 to 2.0-fold ($p<0.001$) compared to 2mM glucose. In the presence of 10^{-5} M CBD, GLP-1 secretion decreased 1.4-fold at 10^{-6} M and 10^{-4} M respectively ($p<0.05$). AM251 was the most potent agonist tested with an EC_{50} value of 5.9×10^{-9} M followed by Abn-CBD ($EC_{50} = 2.4 \times 10^{-6}$ M), OEA ($EC_{50} = 2.1 \times 10^{-6}$ M) and PEA ($EC_{50} = 1.21 \times 10^{-6}$ M).

In pGIPneo STC-1 cells treated with GPR55 agonists, GIP secretion was not affected by either Abn-CBD or AM251 when compared to 2mM glucose. The endogenous agonists OEA and PEA increased GIP secretion 1.1 and 1.7-fold (10^{-6} to 10^{-4} M, $p<0.05$ to $p<0.01$) and 1.1 to 1.8-fold (10^{-8} M to 10^{-6} M, $p<0.05$) respectively. The effect of OEA (10^{-6} M) was reduced 41.1% ($p<0.01$) by 10^{-5} M CBD, while the effect seen by PEA (10^{-6} M) was reduced by 31.0% ($p<0.05$).

All agonists tested demonstrated the ability to increase insulin secretion from pancreatic BRIN-BD11 cells. Synthetic GPR55 agonists Abn-CBD and AM251 increased insulin secretion 1.2 to 2.4-

fold from 10^{-9} to 10^{-4} M ($p<0.05$ - $p<0.001$) and 1.4 to 1.7-fold (10^{-8} - 10^{-4} M, $p<0.01$ - $p<0.001$) respectively in 5.6mM glucose. The endogenous GPR119 agonists OEA and PEA increased insulin secretion in BRIN-BD11 cells from 1.4 to 2.2 fold (10^{-8} - 10^{-4} M, $p<0.05$ - $p<0.001$) and 1.1 to 1.5-fold from 10^{-8} to 10^{-4} M ($p<0.05$ - $p<0.001$) in 5.6mM glucose. In 5.6mM glucose, the most potent agonist tested as determined by EC_{50} was AM251 (5.27×10^{-9} M) followed by Abn-CBD (5.53×10^{-8} M), OEA (2.83×10^{-7} M) and PEA (1.6×10^{-7} M).

Abn-CBD demonstrated a 1.4 to 2.4-fold ($p<0.05$ - $p<0.001$) increase in insulin secretion while AM251 exhibited a 1.4 to 2.2-fold (10^{-7} - 10^{-4} M, $p<0.05$ - $p<0.001$) insulinotropic effect in 16.7mM glucose. OEA increased insulin secretion 1.1 to 1.7-fold (10^{-10} - 10^{-4} M, $p<0.05$ - $p<0.001$) while PEA demonstrated a 1.2 to 1.7-fold increase (10^{-8} - 10^{-4} M, $p<0.05$ - $p<0.001$) compared to 16.7mM glucose. PEA (7.7×10^{-8} M) was the most potent agonist in 16.7mM glucose followed by Abn-CBD (7.83×10^{-7} M) and OEA (1.3×10^{-7} M). AM251 (1.35×10^{-6} M) was the least potent agonist tested in 16.7mM glucose.

GPR55 was found to be co-localised with GLP-1 and PYY in the L cells of the intestine and GIP in the K cells of the intestine as determined by double immunofluorescent staining. Treatment with GPR55 agonists increased GPR55 gene expression in GLUTag cells. AM251 (10^{-4} M) increased GPR55 expression 1.3-fold ($p<0.01$) while Abn-CBD (10^{-4} M) and OEA (10^{-4} M) demonstrated a 1.2-fold ($p<0.05$ to $p<0.01$) increase compared to vehicle control. In male NIH Swiss mouse small intestine, GPR55 expression was reduced 58.9% ($p<0.05$).

GPR55 agonists Abn-CBD and AM251 ($0.1 \mu\text{mol/kg}$ body weight) were administered orally to fasted male Swiss TO mice and increased insulin secretion and lowered blood glucose. Abn-CBD reduced blood glucose 22% ($p<0.001$) and 36.3% ($p<0.001$) after 15 and 30 mins post administration. Administration of Abn-CBD along with GPR55 antagonist CBD confirmed the selectivity of Abn-CBD for GPR55 as insulin secretion was increased 25.2% ($p<0.05$) in the presence of CBD. AM251 reduced blood glucose 34.6% ($p<0.001$) 30 mins post administration compared to glucose. Sitagliptin reduced glycaemic excursion by 24% after 30 mins when administered with glucose. Abn-CBD in combination with Sitagliptin reduced blood glucose reductions of 53.5%, 65.2%, 49.1%, 44.9% and 48.5% ($p<0.001$) 15, 30, 60, 90 and 120 mins post administration. AM251 with Sitagliptin reduced blood glucose 32.0% ($p<0.001$) and 43.9% ($p<0.001$) 15 and 30 mins post administration respectively.

Both Abn-CBD and AM251 increased GLP-1 secretion 15 and 30 mins post administration with increases of 2.1 and 3.2-fold ($p<0.001$) being observed 15 and 30 mins post administration for Abn-CBD and 1.6 and 2.5-fold ($p<0.001$) increases in GLP-1 secretion being observed for AM251. Overall Abn-CBD displayed a 2.2-fold increase in GLP-1 secretion ($p<0.001$) while AM251 increased GLP-1 secretion 1.6-fold ($p<0.001$). Abn-CBD increased GIP secretion 2.5-fold

($p<0.001$) 15 mins post administration while AM251 had no effect. Abn-CBD reduced appetite 23% ($p<0.05$) while AM251 in combination with Sitagliptin reduced appetite 15% ($p<0.05$) Sitagliptin alone had no effect on appetite.

Insulin secretion increased 1.5-fold ($p<0.05$) and 2.6-fold ($p<0.001$) 15 and 30 mins in Swiss TO mice post Abn-CBD administration. Abn-CBD with Sitagliptin increased insulin 2.9-fold increase 30 mins post administration ($p<0.001$). Overall Abn-CBD increased insulin secretion 1.7-fold ($p<0.001$) while Abn-CBD in combination with Sitagliptin increased insulin secretion 1.5-fold ($p<0.001$). AM251 administration increased insulin secretion 1.8-fold ($p<0.01$) and 2.6-fold ($p<0.001$) after 15 and 30 mins respectively while AM251 with Sitagliptin augmented insulin secretion 1.3-fold 30 mins post gavage ($p<0.001$). Overall AM251 increased insulin secretion 3.0-fold ($p<0.001$) while AM251 in combination with Sitagliptin increased insulin secretion 2.6-fold ($p<0.001$).

Streptozotocin induced diabetic Swiss TO mice exhibited hyperglycaemia as the demonstrated a 1.7-fold ($p<0.01$) increase in blood glucose after 18 hrs of fasting and a 2.2-fold ($p<0.001$) increase in non-fasting blood glucose. Higher blood glucose levels were exhibited in streptozotocin induced diabetic mice compared to lean mice during an oral glucose tolerance test with increases of 1.3, 1.6, 1.6, 1.8 and 1.9-fold at 15, 30, 60, 90 and 120 mins post administration respectively ($p<0.05$ - $p<0.001$). The overall increase in blood glucose of streptozotocin induced diabetic mice was 1.6-fold ($p<0.001$) as determined by AUC. Streptozotocin treatment resulted in a 1.1-fold ($p<0.001$) reduction in body weight while lean mice exhibited no reduction.

Streptozotocin induced diabetic mice treated with saline had in increased blood glucose for the duration of the study ($p<0.001$). Daily treatment with Abn-CBD reduced blood glucose 1.4-fold overall ($p<0.01$) while Abn-CBD and Sitagliptin combination therapy resulted in a 1.1-fold reduction ($p<0.05$) in blood glucose. Abn-CBD treatment increased insulin secretion 2.4-fold ($p<0.001$) over the course of the study while Abn-CBD and Sitagliptin combination therapy in a 2.1-fold increase ($p<0.001$). Abn-CBD and Abn-CBD in combination with Sitagliptin increased circulating incretin hormones with overall increases of 1.5 ($p<0.01$) and 1.4-fold ($p<0.01$) being observed for respectively GLP-1. Circulating GIP was increased 1.2-fold ($p<0.001$) in Abn-CBD treated mice while Abn-CBD in combination with Sitagliptin augmented circulating GIP levels 1.3-fold increase ($p<0.001$).

When challenged with glucose, Abn-CBD monotherapy and Abn-CBD combination therapy with Sitagliptin reduced blood glucose 1.4 ($p<0.01$) and 1.5-fold ($p<0.001$) when compared to streptozotocin mice dosed with saline over the course of the study. These treatments also increased insulin secretion over the course of this glucose tolerance test with 1.4-fold ($p<0.001$) increases being observed overall.

Streptozotocin induced diabetic mice had a 1.1-fold increase in body weight compared to lean mice ($p<0.05$). Abn-CBD administration reduced body weight 1.1-fold ($p<0.01$) compared to diabetic mice. Abn-CBD monotherapy or Abn-CBD with Sitagliptin reduced fat mass compared to diabetic mice ($p<0.05$) as well as lean ($p<0.01$). This correlated into a 1.3-fold and a 1.6-fold decrease in body fat percentage compared to lean mice ($p<0.05$).

The research outlined in this study demonstrates that activation of GPR55 plays an important role in glucose homeostasis, β -cell function and incretin hormone regulation. Targeting this receptor with small molecules such as specific FFA agonists that increase enteroendocrine and pancreatic hormone secretion may be a novel therapy for Type 2 Diabetes.

6.2: Introduction

GPR55 is part of the purin receptor cluster of the γ -group of Class A rhodopsin-like GPCRs (Fredriksson *et al.* 2003b). GPR55 was first described in 1999 after its identification from the expressed sequence tags database (Sawzdargo *et al.* 1999). This research described how the GPR55 gene, which is present on chromosome 2q36.3, encodes a receptor consisting of 319 amino acids. It is most closely related to GPR35, GPR23 and purinoceptor P2Y₅. Despite having low sequence homology with the cannabinoid receptors CB1 and CB2 and the lack of the cannabinoid receptor pocket (Petitet, Donlan & Michel 2006), GPR55 is still described as a putative cannabinoid receptor due to its ability to bind atypical cannabinoids (Yang, Zhou & Lehmann 2016). Low sequence identity is common for GPCRs within the purin receptor cluster however GPR55 does share higher sequence homology with a range of GPCRs in this group (Fredriksson *et al.* 2003b). The expression of GPR55 is widespread throughout the body including the brain, dorsal root ganglion neurons, adrenal glands, gastrointestinal tract and pancreas (Sawzdargo *et al.* 1999, Lauckner *et al.* 2008, Ryberg *et al.* 2007, McKillop *et al.* 2013).

GPR55 was recognised as an orphan receptor until the identification of its endogenous ligand lysophosphatidylinositol (LPI) (Oka *et al.* 2007). However the ability of LPI to stimulate the growth of cancer cells in ovarian cancer means its use as a therapeutic aid is limited and other ligands are sought after (Hofmann *et al.* 2015). Three of the first ligands to be identified were Abn-CBD, O1602 and CP55940 (Johns *et al.* 2007, Ryberg *et al.* 2007) closely followed by AM251 and SR141716A (Kapur *et al.* 2009). The search for new ligands has focused on cannabinoids however researchers are now looking further afield with promising results (Heynen-Genel *et al.* 2010). Searching through cannabinoids identified a GPR55 antagonist, CBD, which is highly selective for GPR55 (Sylantsev *et al.* 2013).

GPR55 binds to several different G-proteins as it has been shown to bind to G_{α12} or G_{αq} (Lauckner *et al.* 2008) as well as G_{α13} (Henstridge *et al.* 2009). This results in GPR55 being able to affect several different signalling cascades as it can activate a number of different downstream signalling events such as PLC, RhoA, ROCK, ERK, p38 mitogen activated protein kinase, and Ca²⁺ release (Shore, Reggio 2015). These signalling cascades are thought to be agonist dependent.

As GPR55 is expressed throughout the body it is involved in a wide range of physiological processes. In the intestine GPR55 has been implicated in gut motility (Lin *et al.* 2011), inflammatory bowel diseases (Schicho *et al.* 2010, Włodarczyk *et al.* 2017) and hormone secretion (Harada *et al.* 2017). Knockout of GPR55 leads to increased bone mass and GPR55 antagonism reduced bone resorption thus demonstrating a role for GPR55 in bone formation and thus confirming GPR55 as a receptor for CBD (Whyte *et al.* 2009). Activation of GPR55 by O-1602 lead to reduced colitis and neutrophil recruitment (Schicho *et al.* 2010) while other research has shown GPR55 helps CB2 to recruit neutrophils to sites of inflammation whilst reducing the proinflammatory response mediated by ROS production due to CB2 activation (Balenga *et al.* 2011). GPR55 knockout mice, which had no obvious phenotypic differences from their GPR55^{+/+} littermates, had increased cytokines and reduced response to pain compared to mice with GPR55 (Staton *et al.* 2008). This research demonstrates that GPR55 may play a role in inflammation and also demonstrates a need for further research into the mechanisms behind cannabinoid receptor signalling. Due to the receptor independent effects of cannabinoids on vasculature (White, Hiley 1998) it was suggested that these effects were through another cannabinoid receptor. However it has since been proven that GPR55 has no effect on vasodilation whilst activated by cannabinoids (Johns *et al.* 2007). GPR55 was found to be highly expressed in the MDA-MB-231 breast cancer cell line, with activation by LPI resulting in increased cell motility with this effect being prevented when GPR55 was targeted with siRNA (Ford *et al.* 2010). It has also been shown to contribute to an autocrine loop driving the proliferation of cells in prostate and ovarian cancer cell lines (Pineiro, Maffucci & Falasca 2011) as well as being upregulated in skin cancers (Perez-Gomez *et al.* 2013). Therefore, GPR55 agonists may be novel cancer biomarkers while targeting GPR55 may disrupt the migration and invasiveness of tumours.

Chronic Type 2 Diabetes may lead to increased inflammation and further complications such as nephropathy, neuropathy and retinopathy. GPR55 has previously been shown to have a beneficial effect on inflammation (Staton *et al.* 2008) as well as insulin secretion (McKillop *et al.* 2013, McKillop *et al.* 2016). This has led to the discovery of increased GPR55 expression in disorders such as Crohn's disease or ulcerative colitis (Włodarczyk *et al.* 2017). Previous work carried out in our lab has indicated that activation of GPR55 by Abn-CBD increase incretin hormone secretion and may play a role in glucose homeostasis through both direct and indirect actions on the pancreas (McKillop *et al.* 2016). Antagonism of the cannabinoid receptor CB1 has also previously been shown to be a successful strategy for the reduction of food intake (Reimann, Tolhurst & Gribble

2012) however, due to the serious side effects of rimonabant, other therapeutic targets are sought. As another cannabinoid receptor, GPR55 is therefore an attractive target in metabolic disorders such as Type 2 Diabetes and obesity.

While previous work has focused on the effect of GPR55 on glucose homeostasis through direct action on the pancreas, there are no studies currently published on the effects of GPR55 activation on incretin and intestinal hormone regulation. The effects of GPR55 activation by synthetic (Abn-CBD and AM251) and endogenous agonists (OEA and PEA) on GLP-1 and GIP secretion in both GLUTag and pGIPneo STC-1 cells, respectively, was determined. Agonists were administered in combination with the GPR55 antagonist CBD (10^{-5} M) to assess agonist specificity. The effects of GPR55 agonists on GPR55, proglucagon, GIP and PC1/3 gene expression was also determined in GLUTag cells. The biological effects of Abn-CBD and AM251 alone and in combination with the DPP-IV inhibitor Sitagliptin on pancreatic and intestinal hormone secretion were ascertained in lean and streptozotocin induced diabetic mice, complemented by receptor expression studies.

6.3: Materials and methods

6.3.1: Materials

Abn-CBD (CAY10429, ab144464), OEA (ab141141), PEA (ab120345) and CBD (ab120448) were purchased from Abcam (Cambridge, UK). Multi species GLP-1 total and rat/mouse GIP total ELISA kits were purchased from Merck Millipore (Watford, UK).

6.3.2: Acute hormone secretion from intestinal cell lines

GLUTag or pGIPneo STC-1 cells were seeded into 24-well plates with 150000 cells per well which were incubated overnight in 1ml of culture medium (as described previously in Chapter 2, section 2.1) at 37°C and 5% CO₂. To ensure that cells grew in monolayers, they were pipetted up and down vigorously to ensure proper mixing before seeding. After overnight incubation and preincubation with 1.1mM glucose for 40 mins, acute incubations were carried out at 37°C 5% CO₂ for 2 hrs in 2mM glucose in KRBB buffer supplemented with 10^{-12} to 10^{-4} M concentrations of Abn-CBD, AM251, OEA and PEA. After incubations, supernatants (950µl) were collected and frozen at -20°C until ELISA could be carried out as described in Chapter 2, section 2.3.

6.3.3: Acute insulin secretion from pancreatic BRIN-BD11 cells

Cells were seeded in 24-well plates with 150000 BRIN-BD11 cells per well and incubated overnight in 1ml of RPMI 1640 media at 37°C and 5% CO₂. Following this incubation, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹² to 10⁻⁴M concentrations of Abn-CBD, AM251, OEA and PEA with 10mM alanine as a positive control. After incubations, supernatants (950µl) were collected and frozen at -20°C until radioimmunoassay could be carried out as described in Chapter 2, section 2.2.

6.3.4: Acute effects of GPR55 on cell toxicity

Cells were seeded in 96-well plates with 40000 cells per well of GLUTag, pGIPneo STC-1 or BRIN-BD11 cells and incubated overnight in cell culture media (as described in Chapter 2, section 2.1) in an atmosphere of 5% CO₂ at 37°C. After 20hrs, BRIN-BD11 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of GPR55 agonist with 1mM H₂O₂ as a cytotoxic control (n=3). After 48hrs, GLUTag and pGIPneo STC-1 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 2 hrs in 2mM in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of GPR55 agonists with 1mM H₂O₂ as a cytotoxic control (n=3). After incubation, supernatant was decanted, cells were washed with HBSS and 0.5mg/ml MTT was added for 2 hrs. Following MTT incubation, supernatant was discarded and DMSO was added to solubilise crystals. Optical density was subsequently measured at 570nm and 630nm using the Flexstation 3 (Molecular Devices, CA, USA).

6.3.5: Immunofluorescence staining in GLUTag, pGIPneo STC-1, STC-1 cells and lean and HFF NIH Swiss mouse small intestine

GLUTag, pGIPneo STC-1 and STC-1 cells were seeded at a density of 40000 cells on to polylysine coated slides overnight at 37°C in an atmosphere of 95% air and 5% CO₂ as described in Chapter 2, section 2.1. Swiss TO mice were anaesthetised by isoflurane and killed by cervical dislocation. Pancreas and intestine was excised as described in Chapter 2, section 2.6.3. Immunohistochemistry was carried out by incubating with rabbit polyclonal anti-GPR55 and goat polyclonal anti-GLP-1, goat polyclonal anti-GIP or goat polyclonal anti-PYY with the dilutions outlined in Chapter 2, Table 2.1.

6.3.6: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Chapter 2, Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH.

6.3.7: Acute *in vivo* oral glucose tolerance tests

Male Swiss TO mice were housed as described in Chapter 2, section 2.7. The effects GPR55 agonists Abn-CBD and AM251 (0.1µmol/kg BW), as well as in combination with Sitagliptin phosphate (50mg/kg BW) or CBD (0.1µmol/kg BW) in lean male Swiss TO mice were investigated. Before test compounds were administered mice were fasted for 18 hrs and blood subsequently collected (t=0). Test compounds were administered orally in glucose (18mmol/kg BW), and blood was collected at 15, 30, 60, 90 and 120 mins along with blood glucose measurements. Collected blood was centrifuged at 13200rpm for 5 mins at 4°C using a Beckman centrifuge (Beckman Instruments, UK). Plasma was stored at -20°C until hormone measurements by RIA or ELISA as described in Chapter 2, section 2.3.

6.3.8: Multiple low dose streptozotocin induced-diabetic mice

Age-matched (26 wks), male Swiss TO mice were sourced from Envigo (Huntingdon, UK). Mice were housed in single cages and were supplied drinking water and standard rodent maintenance diet (60% carbohydrate, 30% protein, 10% fat (12.99 kJ/g), Trouw Nutrition, Cheshire, UK) *ad libitum*. Diabetes was chemically induced in 4 hr fasted Swiss TO mice through 4 intraperitoneal injections of streptozotocin (40mg/kg body weight, 0.1M sodium citrate, pH 4.5) which were received on alternate days. Diabetes was confirmed through an oral glucose tolerance test (OGTT) which was performed 14 days after the first injection of streptozotocin injection (day 0) on 18 hr fasted Swiss TO mice.

6.3.9: Treatment procedure and parameters assessed

The long term effects of oral Abn-CBD (0.1 μ mol/kg BW) or saline (0.9% w/v NaCl) in multiple low dose streptozotocin-induced diabetic Swiss TO mice (n=6) was assessed over a period of 21 days. Hyperglycaemia was confirmed in diabetic mice through an oral glucose tolerance test which was performed 14 days after the first streptozotocin injection (day 0) on 18 hr fasted NIH Swiss mice as previously described (Chapter 2, section 2.8.2). Fluid intake, food intake, body weight, non-fasted blood glucose, insulin, GLP-1 and GIP concentrations were monitored every 3 days. Blood was collected via tail vein bleeding of conscious mice and was collected and analysed for blood glucose, insulin, GLP-1 and GIP as described in Chapter 2, section 2.8.4. After the testing period, glucose tolerance tests (18mmol/kg body weight) were carried out to assess glucose tolerance on 18 hr fasted streptozotocin treated mice and Swiss TO mice on standard rodent maintenance diet as outlined in Chapter 2, Section 2.8.2.

6.3.10: Measurement of plasma hormones

Blood was collected via tail vein bleed on conscious mice every 3 days for biochemical analysis on a variety of hormones. Blood glucose determination was performed using a Bayer Contour Next meter (Leverkusen, DE) every 3 days. Plasma insulin was quantified using insulin RIA as previously described in Chapter 2, section 2.2. Rat/mouse GIP total ELISA kit (Millipore, Watford, UK) was used to measure total GIP concentrations in mouse plasma following chronic treatment. Multi species GLP-1 total ELISA was used to measure total GLP-1 content of mouse plasma. These sandwich based ELISAs are described in detail in Chapter 2, section 2.3.

6.3.11: Insulin sensitivity

Prior to the injection of insulin, blood glucose was measure in non-fasted Swiss TO mice via tail vein bleeding using the Bayer Contour glucose meter. Bovine insulin (25U/kg) in 0.9% saline was administered using intraperitoneal injection of bovine insulin (25U/kg) and blood glucose was monitored at 30 and 60 mins post injection.

6.3.12: Dual energy X-ray absorption (DEXA)

Non-fasted Swiss TO mice were culled by cervical dislocation after being anaesthetised using isoflurane, and subsequently arranged on the specimen tray to ensure both limbs and tail were extended away from the body. In order to ensure calibration and quality control, 25 measures of the

aluminium/lucite phantom (0.069g/cm², 12.0% fat) were taken using a Lunar PIXImus Dual energy X-ray absorption (DEXA) system (software version 1.4x) before any specimens were measured. DEXA scanning was performed on all carcasses as per manufacturer's guidelines. The Lunar PIXImus DEXA system measures percentage body fat as well as bone mineral density (BMD, g/cm³) and bone mineral content (BMC, g) using a fully integrated densitometer.

6.3.13: Assessment of plasma lipid profiles

Plasma triglyceride and HDL cholesterol concentrations were measured using an I-lab 650 clinical chemistry instrument (Instrumentation Laboratory, Warrington, UK).

6.4: Results

6.4.1: Effects of GPR55 agonists and antagonist CBD on GLP-1 secretion from intestinal GLUTag cells

The effects of endogenous GPR55 agonists OEA and PEA and synthetic agonists Abn-CBD and AM251 along with GPR55 antagonist CBD on GLP-1 secretion from GLUTag cells was investigated in 2mM glucose. Synthetic agonist Abn-CBD demonstrated a 1.7 to 2.4-fold (10^{-8} - 10^{-4} M, $p < 0.05$) increase in GLP-1 secretion from GLUTag cells compared to 2mM glucose basal control (Figure 6.1A). When tested in combination with the GPR55 agonist CBD (10^{-5} M), GLP-1 secretion was reduced to basal levels with a 1.4 to 1.7-fold (10^{-8} - 10^{-4} M, $p < 0.05$) decrease in secretion being observed.

The other synthetic GPR55 agonist, AM251, increased GLP-1 secretion 1.4 to 1.7-fold (10^{-8} M to 10^{-6} M, $p < 0.01$ - $p < 0.001$) compared to basal control (Figure 6.2A). The addition of 10^{-5} M CBD decreased GLP-1 secretion at all concentrations tested. The largest decrease in GLP-1 secretion was demonstrated at 10^{-6} M and 10^{-8} M AM251, where a 2.1-fold decrease in secretory ability was detected ($p < 0.01$ to $p < 0.001$). At the lowest concentration of AM251 tested (10^{-10} M), there was a 1.7-fold decrease in GLP-1 secretion compared to the absence of CBD ($p < 0.01$).

PEA (Figure 6.4A) was the most potent endogenous GPR55 agonist in augmenting GLP-1 secretion. GLP-1 secretion from GLUTag cells was increased 1.3 to 2.5-fold compared to basal control (10^{-8} M to 10^{-4} M, $p < 0.01$ to $p < 0.001$), with the addition of 10^{-5} M CBD reducing the effect of PEA agonism by 1.6, 1.4 and 1.9-fold ($p < 0.05$ to $p < 0.001$)

The other endogenous GPR55 agonist tested, OEA (10^{-6} to 10^{-4} M), promoted GLP-1 secretion 1.8 to 2.0-fold ($p < 0.001$) compared to 2mM glucose (Figure 6.3A). In the presence of 10^{-5} M CBD, GLP-1 secretion decreased 1.4-fold compared to OEA treatment alone at 10^{-6} M and 10^{-4} M respectively ($p < 0.05$).

AM251 was the most potent agonist tested with an EC_{50} value of 5.9×10^{-9} M. The other synthetic agonist, Abn-CBD ($EC_{50} = 2.4 \times 10^{-6}$ M), was more potent than the endogenous agonists tested. The most potent endogenous agonist was OEA ($EC_{50} = 2.1 \times 10^{-6}$ M) with PEA the least potent of all agonists ($EC_{50} = 1.21 \times 10^{-6}$ M). All agonists tested showed no adverse effect on cell viability as measured by MTT assay (Figures 6.1B-6.4B).

6.4.2: Effects of GPR55 agonists and antagonist CBD on GIP secretion from intestinal pGIPneo STC-1 cells

Intestinal pGIPneo STC-1 cells were treated with both synthetic, Abn-CBD (Figure 6.5A) and AM251 (Figure 6.6A), and endogenous, OEA (Figure 6.7A) and PEA (Figure 6.8A), GPR55 agonists to assess the effect of GPR55 activation on GIP secretion from the K cells of the intestine. Both synthetic agonists tested demonstrated no ability to increase GIP secretion compared to the vehicle control (2mM glucose). The endogenous agonist OEA (Figure 6.7A) increased GIP secretion 1.1 and 1.7-fold (10^{-6} to 10^{-8} M, $p < 0.05$ to $p < 0.01$). In the presence of 10^{-5} M CBD, this effect was reduced 41.1% ($p < 0.01$). PEA promoted the secretion of GIP from pGIPneo STC-1 cells as a 1.1 to 1.8-fold increase was observed (10^{-6} M to 10^{-4} M, $p < 0.05$). In the presence of GPR55 antagonist 10^{-5} M CBD, the effect of PEA (10^{-4} M) on GIP secretion was reduced by 31.0% ($p < 0.05$) (Figure 6.8A). All agonists tested demonstrated no cytotoxic effects as measured by MTT assay (Figures 6.5B-6.8B). Interestingly, both Abn-CBD (10^{-6} M to 10^{-4} M, Figure 6.5B) and AM251 (10^{-8} M to 10^{-4} M, Figure 6.6B) demonstrated an increased K-cell viability ($p < 0.05$).

6.4.3: Effects of GPR55 agonists on insulin secretion from pancreatic BRIN-BD11 cells

The insulinotropic ability of the synthetic GPR55 agonists Abn-CBD and AM251, as well as endogenous agonists OEA and PEA were tested at both 5.6 and 16.7mM glucose in pancreatic BRIN-BD11 cells (Figures 6.9A-6.16A). In normoglycaemic conditions synthetic agonist, Abn-CBD (Figure 6.9A), increased insulin secretion 1.2 to 2.4-fold from 10^{-9} to 10^{-4} M ($p < 0.05$ - $p < 0.001$). The other synthetic GPR55 agonist, AM251 (Figure 6.11A), increased insulin secretion 1.4 to 1.7-fold (10^{-8} - 10^{-4} M, $p < 0.01$ - $p < 0.001$). Endogenous agonist OEA (Figure 6.13A) increased insulin secretion from 1.4 to 2.2 fold in a dose dependent manner (10^{-8} - 10^{-4} M, $p < 0.05$ - $p < 0.001$). PEA (Figure 6.15A) increased insulin secretion 1.1 to 1.5-fold from 10^{-8} to 10^{-4} M ($p < 0.05$ - $p < 0.001$).

In hyperglycaemic conditions Abn-CBD (Figure 6.10A) increased secretion of insulin 1.4 to 2.4-fold ($p<0.05$ - $p<0.001$) while AM251 (Figure 6.12A) exhibited a 1.4 to 2.2-fold (10^{-7} - 10^{-4} M, $p<0.05$ - $p<0.001$) insulinotropic effect. OEA (Figure 6.14A) increased insulin secretion 1.1 to 1.7-fold (10^{-10} - 10^{-4} M, $p<0.05$ - $p<0.001$) compared to 16.7mM glucose, while PEA (Figure 6.16A) demonstrated a 1.2 to 1.7-fold increase (10^{-8} - 10^{-4} M, $p<0.05$ - $p<0.001$).

The potency of all agonists tested was determined by calculation of the EC_{50} value (Figure 6.17A and 6.17B). The most potent agonist tested in 5.6mM glucose was AM251 (5.27×10^{-9} M), closely followed by another synthetic agonist, Abn-CBD (5.53×10^{-8} M). The endogenous agonists tested had very similar potencies for GPR55 in normal glucose conditions with OEA (2.83×10^{-7} M) being more potent than PEA (1.6×10^{-7} M). In hyperglycaemic conditions PEA demonstrated the highest potency (7.7×10^{-8} M) while AM251 was the least potent agonist tested (1.35×10^{-6} M). Abn-CBD (7.83×10^{-7} M) was again more potent than OEA (1.3×10^{-7} M). Apart from PEA, all agonists tested had lower potency in hyperglycaemic conditions compared to 5.6mM glucose. All agonists tested also demonstrated no significant effects on cell viability or proliferation (Figures 6.9B-6.16B) at both concentrations of glucose tested.

6.4.4: Distribution of GPR55 and GLP-1 in intestinal GLUTag cells and lean and HFF NIH Swiss mouse small intestine

The localisation of GPR55 in the L-cells of the intestine was examined through immunohistochemistry in both the intestinal GLUTag cell line and in NIH Swiss mice which have been fed either a lean or HFF diet. GLP-1 was expressed throughout GLUTag cells (Figure 6.18B) and was also present in the L-cells of the small intestine of both lean and HFF NIH Swiss mice (Figure 6.19B and 6.20B). The presence of GPR55 in GLUTag cells and lean and HFF NIH Swiss mice was also confirmed (Figure 6.18C, Figure 6.19C and Figure 6.20C). The combined fluorescence of both GLP-1 and GPR55 demonstrated the co-localisation of GPR55 and GLP-1 in intestinal L-cells (Figure 6.18D, Figure 6.19D and Figure 6.20D). qPCR confirmed the presence of GPR55 in both lean and HFF mouse small intestinal tissue (Figure 6.21), with a 2.5-fold decrease in GPR55 gene expression observed in mice on a HFF diet ($p<0.05$).

6.4.5: Distribution of GPR55 and GIP in intestinal pGIPneo STC-1 cells and lean and HFF NIH Swiss mouse small intestine

The cellular localisation of GPR55 and GIP in the K cells of the intestine was investigated through the immunohistochemical staining of pGIPneo STC-1 cells (Figure 6.22) and lean and HFF NIH Swiss mice (Figures 6.23 and 6.24). GPR55 was found to be present in the pGIPneo STC-1 cells

(Figure 6.22C). GIP was found to be present in both lean and HFF NIH Swiss mouse small intestine (Figures 6.23B and 6.24B), as was GPR55 (Figures 6.23C and 6.24C). A merge of both GPR55 and GIP confirmed the presence of both entities in the same K-cell (Figure 6.23D and 6.24D).

6.4.6: Distribution of GPR55 and PYY in intestinal STC-1 and lean and HFF NIH Swiss mouse small intestine

The presence of PYY and GPR55 in STC-1 cells (Figure 6.25) was determined through immunohistochemistry. PYY (Figure 6.25B) and GPR55 (Figure 6.25C) were found throughout STC-1 cells and combining the fluorescences of both images showed large areas of co-localisation of GPR55 and PYY (Figure 6.25D). GPR55 and PYY were also co-localised in both lean and HFF small intestine from NIH Swiss mice (Figures 6.26D and 6.27D).

6.4.7: Effects of GPR55 agonists on GPR55 gene expression in intestinal GLUTag cells

The effects of 2mM glucose and GPR55 agonists on GPR55 gene expression were examined using qPCR. (Figure 6.28A). AM251 (10^{-4} M) increased GPR55 expression 1.3-fold ($p<0.01$) while AM251 and OEA up-regulated GPR55 gene expression 1.2-fold ($p<0.05$ to $p<0.01$) compared to 2mM glucose vehicle control.

6.4.8: Acute effects of GPR55 agonists on incretin hormones in male Swiss TO mice

Mice were orally gavaged in this study in order to study the effects of GPR55 agonists on both GLP-1 and GIP secretion. Both Abn-CBD and AM251 increased GLP-1 secretion 15 and 30 mins post administration (Figure 6.29A). Abn-CBD activation of the GPR55 receptor generated the highest effect on GLP-1 secretion, with a 2.1 and 3.2-fold increase 15 and 30 mins post administration respectively ($p<0.001$). AM251 increased GLP-1 secretion 1.6 and 2.5-fold at 15 and 30 mins post administration ($p<0.001$). Overall Abn-CBD displayed a greater effect on GLP-1 secretion as determined by AUC (Figure 6.29B) with a 2.2-fold increase being observed ($p<0.001$) while AM251 increased GLP-1 secretion 1.6-fold overall ($p<0.001$).

Of both GPR55 agonists tested, only Abn-CBD increased GIP secretion (Figure 6.30A). At 15 mins post administration GIP secretion was increased 2.45-fold ($p<0.001$) before returning to basal levels 30 mins post administration. AM251 provided no significant effect on GIP secretion. Overall Abn-CBD increased GIP secretion 2.1-fold ($p<0.001$) as determined by AUC (Figure 6.30B).

6.4.9: Acute effects of GPR55 agonists on blood glucose and insulin in male Swiss TO mice

Abn-CBD and AM251 were administered to fasted male Swiss TO mice via oral gavage at concentrations of 0.1 $\mu\text{mol/kg}$ body weight. Abn-CBD (Figure 6.31A) displayed a greater effect on reducing blood glucose *in vivo* compared to AM251 (Figure 6.32A). Blood glucose was reduced by 22% at 15 mins after Abn-CBD administration ($p < 0.05$) and 36.3% after 30 mins ($p < 0.001$). Administration of Abn-CBD along with GPR55 antagonist CBD (0.1 $\mu\text{mol/kg}$ BW) increased blood glucose 25.2% 15 mins post administration ($p < 0.05$). AM251 (Figure 6.32A) reduced blood glucose 34.6% ($p < 0.001$) 30 mins post administration compared to glucose.

The DPP-IV inhibitor Sitagliptin reduced glycaemic excursion by 24% after 30 mins when administered with glucose. When Abn-CBD and Sitagliptin were administered in combination (Figure 6.31A), blood glucose was markedly reduced at 15, 30, 60, 90 and 120 mins post administration with reductions of 53.5%, 65.2%, 49.1%, 44.9% and 48.5% ($p < 0.001$). AM251 in combination with Sitagliptin (Figure 6.32A) reduced blood glucose 32.0% ($p < 0.001$) and 43.9% ($p < 0.001$) 15 and 30 mins post administration respectively.

Abn-CBD administration alone increased insulin secretion 1.5-fold ($p < 0.05$) 15 mins post administration (Figure 6.33A) with this effect sustained 30 mins post gavage with an increase of 2.6-fold ($p < 0.001$) being observed. Abn-CBD in combination with Sitagliptin resulted in a 2.9-fold increase on insulin secretion 30 mins post administration ($p < 0.001$). Overall Abn-CBD increased insulin secretion 1.7-fold ($p < 0.001$) while Abn-CBD in combination with Sitagliptin increased insulin secretion 1.5-fold ($p < 0.001$) as determined by AUC (Figure 6.33B).

Oral administration of AM251 (Figure 6.34A) reduced blood glucose 34.6% 30 mins post administration ($p < 0.01$). When administered in combination with Sitagliptin, blood glucose was reduced by 30% after 15 mins ($p < 0.01$). This effect was continued after 30 mins when blood glucose was reduced 44% ($p < 0.001$). The effects on blood glucose were reduced when AM251 was administered alongside the GPR55 antagonist CBD (0.1 $\mu\text{mol/kg}$ BW). Insulin secretion increased 1.78-fold ($p < 0.01$) and 2.6-fold ($p < 0.001$) 15 and 30 mins post administration respectively (Figure 6.34A) in mice administered AM251. When AM251 was administered in combination with Sitagliptin, insulin was increased 1.3-fold 30 mins post gavage ($p < 0.001$) compared to glucose administration alone. Overall AM251 increased insulin secretion 3.0-fold ($p < 0.001$) while AM251 in combination with Sitagliptin increased insulin secretion 2.6-fold ($p < 0.001$) as determined by AUC (Figure 6.34B).

6.4.10: Acute effects of GPR55 agonists on feeding

Male Swiss TO mice were trained to eat for 3 hrs daily and the effects of oral administration of GPR55 agonists and Sitagliptin on these mice was tested. Abn-CBD (0.1 μ mol/kg BW) reduced appetite by 23% ($p < 0.05$) when administered alone (Figure 6.35A). Sitagliptin alone had no effect on appetite. Oral administration of AM251 (0.1 μ mol/kg BW) in combination with Sitagliptin was able to reduce appetite 15% ($p < 0.05$) after 3 hrs (Figure 6.35B). The administration of AM251 alone produced no significant effect.

6.4.11: Effects of multiple low dose streptozotocin on blood glucose and plasma insulin in male Swiss TO mice

The blood glucose of lean and multiple low dose streptozotocin treated mice was observed for 14 days after initial injection of streptozotocin (Figure 6.36).

Lean mice underwent no changes in blood glucose for the duration of saline injections. However streptozotocin caused an initial 38% decrease in blood glucose 2 days after the first streptozotocin injection ($p < 0.001$). By the last streptozotocin injection mice had increased blood glucose compared to lean mice at the same time point ($p < 0.01$) however there was no increase compared to day 1 blood glucose levels. Four days after the last injection blood glucose had increased in streptozotocin treated mice 1.5-fold ($p < 0.001$) compared to initial levels.

Both lean mice and mice treated with multiple low dose streptozotocin were fasted for 18 hrs and underwent an oral glucose tolerance test 15 days after initial injection of streptozotocin (Figure 6.36A). After 18 hrs of fasting streptozotocin induced diabetic mice had increased blood glucose with an increase of 1.7-fold being observed ($p < 0.01$). Oral administration of 18mmol/kg BW glucose lead to increased blood glucose in both lean and streptozotocin induced mice. Throughout the course of the test streptozotocin treated mice had higher blood glucose levels than lean mice with increases of 1.3, 1.6, 1.6, 1.8 and 1.9-fold at 15, 30, 60, 90 and 120 mins post administration respectively ($p < 0.05$ - $p < 0.001$). The overall increase in blood glucose of streptozotocin induced diabetic mice was 1.6-fold ($p < 0.001$) as determined by AUC (Figure 6.35B).

Streptozotocin induced diabetic mice also had increased non-fasting blood glucose compared to lean mice with a 2.2-fold ($p < 0.001$) increase in being demonstrated (Figure 6.37A). Before treatment, streptozotocin treated mice were split into groups with no significant difference in blood glucose levels (Figure 6.37B).

Streptozotocin treatment resulted in a reduction in body weight (Figure 6.39A) with a 1.1-fold decrease being observed on the last day of streptozotocin injection ($p < 0.01$) and a 1.1-fold decrease

being observed 14 days after the first injection ($p<0.001$). Lean mice had no reduction in body weight (Figure 6.39B) while the reduction in body weight in streptozotocin treated mice did not result in significant body weight differences with lean mice before grouping (Figure 6.40A). After grouping, streptozotocin induced diabetic mice had no significant difference in body weight (Figure 6.40B).

6.4.12: Effects of Abn-CBD on non-fasting GIP and GLP-1 levels

Daily oral administration of Abn-CBD or Abn-CBD in combination with Sitagliptin resulted in increased circulating incretin hormones. Abn-CBD demonstrated an earlier effect on GLP-1 than Abn-CBD and with a 1.6-fold increase being observed after 12 days of treatment (Figure 6.44A, $p<0.001$). After 21 days Abn-CBD in combination with Sitagliptin had increased circulating GLP-1 levels 2.0-fold ($p<0.001$) while Abn-CBD administration had increased GLP-1 levels 2.1-fold. Overall Abn-CBD and Sitagliptin had the greatest effect as determined by AUC with an overall increase in GLP-1 levels of 1.5-fold (Figure 6.44B, $p<0.01$) while Abn-CBD demonstrated an increase of 1.4-fold ($p<0.01$).

As seen with GLP-1 levels, Abn-CBD combined with Sitagliptin increased GIP levels earlier than Abn-CBD treatment alone (Figure 6.45A). After 12 days of administration, Abn-CBD and Sitagliptin had increased GIP levels 1.8-fold ($p<0.01$). After 21 days of administration Abn-CBD in combination with Sitagliptin produced an increase of 2.0-fold compared to diabetic mice ($p<0.001$) while Abn-CBD administration resulted in a 1.7-fold increase ($p<0.001$). The overall increase demonstrated by Abn-CBD and Sitagliptin was 1.3-fold compared to streptozotocin induced diabetic mice ($p<0.05$). Abn-CBD treatment alone resulted in a 1.2-fold increase ($p<0.01$). Interestingly streptozotocin induced diabetic mice had a 1.3-fold increase in GIP levels compared to lean mice.

6.4.13: Effects of GPR55 agonist Abn-CBD on body weight, fluid intake, non-fasting blood glucose and plasma insulin

Chronic administration of Abn-CBD monotherapy and Abn-CBD combination therapy with Sitagliptin had no effect on body weight throughout the study (Figure 6.41A). Abn-CBD monotherapy reduced appetite after 18 days of treatment with a 27.7% ($p<0.001$) reduction being observed at this stage compared to diabetic mice treated with saline (Figure 6.42B). This effect was continued until the end of the study where Abn-CBD treated mice exhibited a 29.0% reduction ($p<0.001$) in appetite. Abn-CBD combination therapy with Sitagliptin also reduced appetite after 18 days of treatment. This therapy produced reductions of 28.8% and 32.2% ($p<0.001$) after 18 and

21 days of treatment respectively compared to streptozotocin induced diabetic mice. Abn-CBD combination therapy was the only treatment that reduced fluid intake (Figure 6.43) compared to streptozotocin induced diabetic mice as it exhibited a reduction of 46.3% ($p<0.05$).

Compared to lean mice, streptozotocin resulted in increased blood glucose for the duration of the study (Figure 6.46A, $p<0.001$). Daily treatment with Abn-CBD reduced blood glucose 1.4-fold overall (Figure 6.46B, $p<0.01$) while treatment with Abn-CBD and Sitagliptin resulted in a 1.12-fold reduction ($p<0.05$). These results were confirmed as both Abn-CBD and Abn-CBD in combination increased insulin secretion from streptozotocin induced diabetic mice (Figure 6.47A). Overall Abn-CBD treatment increased insulin secretion 2.4-fold ($p<0.001$) while Abn-CBD in combination with Sitagliptin resulted in a 2.1-fold increase (Figure 6.47B, $p<0.001$).

6.4.14: Effects of Abn-CBD on glucose tolerance and insulin sensitivity

Daily oral administration of Abn-CBD or Abn-CBD in combination with Sitagliptin resulted in positive effects on glucose tolerance (Figure 6.48A). When administered glucose orally, mice who were treated with Abn-CBD had an overall reduction of 1.4-fold when compared to streptozotocin mice dosed with saline over the course of the study (Figure 6.48B, $p<0.01$). Like Abn-CBD treatment alone, Abn-CBD in combination also had a reducing effect on hyperglycaemia with a decrease in blood glucose of 1.5-fold being observed overall during the glucose tolerance test ($p<0.001$).

Both Abn-CBD and Abn-CBD in combination with Sitagliptin increased insulin secretion when compared to streptozotocin mice over the course of the glucose tolerance test. Abn-CBD demonstrated a 1.6-fold increase in insulin secretion 15 mins after glucose had been administered (Figure 6.49A, $p<0.001$) and the same effect was observed with Abn-CBD and Sitagliptin which demonstrated a 1.6-fold increase in insulin secretion at the same time point ($p<0.001$). Lean mice demonstrated increased insulin secretion compared to streptozotocin induced diabetic mice treated with saline at all time points except for 60 mins post administration ($p<0.01$ to $p<0.001$). Overall both Abn-CBD ($p<0.001$) and Abn-CBD combined with Sitagliptin ($p<0.01$) increased insulin secretion 1.4-fold compared to streptozotocin treated mice (Figure 6.49B). When injected with insulin both Abn-CBD and Abn-CBD in combination with Sitagliptin reduced blood glucose compared to streptozotocin induced diabetic mice (Figure 6.50B).

6.4.15: Effects of Abn-CBD as measured by DEXA

Of all the groups analysed, mice with streptozotocin induced diabetes had the highest body weight and this was the only group which was increased 1.1-fold compared to lean mice (Figure 6.52A, $p < 0.05$). Daily oral administration of Abn-CBD resulted in a 1.1-fold reduction in body weight ($p < 0.01$) compared to diabetic mice treated with saline, however this effect was not maintained when administered in combination with Sitagliptin. No significant effects were observed in lean mass (Figure 6.52B) however both groups of mice treated with Abn-CBD or Abn-CBD with Sitagliptin had reduced fat mass (Figure 6.53A, $p < 0.05$) compared to diabetic mice as well as lean ($p < 0.01$). This correlated into a 1.3-fold and a 1.6-fold decrease in body fat percentage compared to lean mice (Figure 6.53B, $p < 0.05$).

6.4.16: Effects of chronic Abn-CBD treatment on lipid profiles

Streptozotocin treated mice had reduced triglycerides compared to lean mice (Figure 6.56A). Diabetic mice treated with saline exhibited a 2.9-fold ($p < 0.001$) decrease in triglycerides compared to lean mice with decreases of 2.3-fold ($p < 0.001$) and 1.7-fold ($p < 0.001$) being observed in mice treated with Abn-CBD monotherapy and combination therapy respectively. Streptozotocin induced diabetes had no effect on total cholesterol nor had Abn-CBD monotherapy or in combination with Sitagliptin (Figure 6.56B). HDL cholesterol was also not affected (Figure 6.57B). Induction of diabetes with streptozotocin reduced LDL levels with saline treated mice having a 1.6-fold ($p < 0.05$) reduction compared to lean mice (Figure 6.57A). Diabetic mice treated with Abn-CBD combination therapy exhibited a 1.9-fold ($p < 0.05$) reduction in LDL compared to lean mice. Abn-CBD monotherapy reduced LDL levels 2.92-fold ($p < 0.001$) compared to lean mice and this was significantly lower than the LDL levels observed in streptozotocin induced diabetic mice treated with saline with a reduction of 45.1% being observed ($p < 0.05$).

6.5: Discussion

GPR55 is a novel cannabinoid receptor which has previously been shown to increase intracellular calcium through $G_{\alpha 12}$ and $G_{\alpha q}$ and it has also been shown to be coupled to $G_{\alpha 13}$ (Ryberg *et al.* 2007). Due to its widespread expression throughout the body, GPR55 has been proposed to play a role in various physiological processes and has been proposed as a new novel anticancer target (Leyva-Illades, DeMorrow 2013). This receptor has also demonstrated insulinotropic activity both *in vitro* (McKillop *et al.* 2013) and *in vivo* (McKillop *et al.* 2016) and it may play a regulatory role on glucagon secretion (Liu *et al.* 2016). Therefore the effects of GPR55 activation on glucose homeostasis make it an interesting target for the treatment of Type 2 Diabetes.

This study investigated the effects of GPR55 agonists on insulin, GLP-1, GIP and PYY secretion. In BRIN-BD11 cells, all agonists tested demonstrated a dose dependent insulinotropic effect in both normal and hyperglycaemic conditions. The most potent agonist tested in normal conditions was AM251 followed by Abn-CBD then the endogenous agonists OEA and PEA as per the EC₅₀ value for each agonist. In hyperglycaemic conditions PEA became the most potent agonist whilst AM251 was the least potent. Abn-CBD was again more potent than OEA but less potent than PEA. Abn-CBD demonstrated the greatest insulinotropic effect followed by OEA, AM251 and PEA in normal conditions. In hyperglycaemic conditions, Abn-CBD again had the greatest insulinotropic effect followed by AM251, OEA and PEA. This confirms the previous results which have demonstrated that activation of GPR55 can regulate glucose homeostasis through insulin secretion (McKillop *et al.* 2016, McKillop *et al.* 2013, Liu *et al.* 2016).

GPR55 agonist LPI has previously demonstrated that activation of GPR55 increases GLP-1 secretion from GLUTag cells (Liu *et al.* 2016). In this study, immunohistochemistry confirmed the co-localisation of GPR55 and GLP-1 in GLUTag cells confirming earlier work showing the presence of GPR55 in GLUTag cells (Liu *et al.* 2016). Abn-CBD has also previously been shown to increase GLP-1 secretion after long term administration to mice with streptozotocin induced diabetes (McKillop *et al.* 2016) and it demonstrated the greatest effect on GLP-1 release from GLUTag cells while being more potent than both OEA and PEA based on EC₅₀ values. While displaying greater potency than Abn-CBD, AM251 demonstrated a smaller increase in GLP-1 secretion. Of the endogenous agonists tested, OEA demonstrated the greatest effect on GLP-1 secretion as well as having the highest potency. PEA had no effect on GLP-1 secretion. GPR55 antagonist CBD was used to determine selectivity of the tested agonists for GPR55. All agonists tested demonstrated selectivity for GPR55. Abn-CBD and AM251 demonstrated selectivity at all concentrations tested, while OEA demonstrated selectivity for GPR55 at concentrations which increased GLP-1 secretion. PEA only demonstrated selectivity at the lowest concentration which was investigated.

The determination of proglucagon gene expression from GLUTag cells helps to confirm/validate these findings. Both Abn-CBD and AM251 as well as OEA increase proglucagon gene expression while PEA demonstrates no effect. In order for more GLP-1 to be secreted at the protein level, more GLP-1 needs to be expressed during mRNA synthesis. These results suggest that GPR55 plays a regulatory role in GLP-1 secretion.

The effects of GPR55 agonists on GIP secretion from pGIPneo STC-1 cells was studied and demonstrated that both Abn-CBD and AM251 had no effect on the release of GIP. Immunohistochemistry in primary tissue confirmed the co-localisation of both GIP and GPR55 *ex*

vivo. Both OEA and PEA increased GIP secretion although these agonists have the ability to work through other receptors that may be present in these cells (Moran *et al.* 2014a).

As the agonists tested had demonstrated an ability to increase intestinal hormone secretion, their effects *in vivo* was tested orally in lean Swiss TO mice. Abn-CBD demonstrated the greatest effects on hyperglycaemia. When administered in combination with Sitagliptin, a DPP-IV inhibitor which is also administered orally, both agonists reduced hyperglycaemia. This confirmed previous results demonstrating that oral administration of Abn-CBD played a role in glucose homeostasis (McKillop *et al.* 2016). Previous work has demonstrated that cannabinoid receptors may help to potentiate GLP-1 receptor activation (Radziszewska, Bojanowska 2012) and using knock out mice models it has been shown that the effects of Abn-CBD on glucose homeostasis are diminished in the absence of incretin hormone receptors (McKillop *et al.* 2016). The work carried out in this study demonstrates that both Abn-CBD and AM251 can increase both GLP-1 and GIP secretion acutely and long term. It also confirms the previous work which has shown that GPR55 agonists can regulate glucose homeostasis (McKillop *et al.* 2016, McKillop *et al.* 2013).

The CB1 cannabinoid receptors has been shown to increase appetite while antagonism of this receptors can lead to reduced appetite (Wiley *et al.* 2005) whereas activation of CB2 can reduce appetite in mice (Verty *et al.* 2015). In order to test the effects of GPR55 activation on appetite, mice were trained to eat for 3 hrs and orally administered with a GPR55 agonist before eating. Abn-CBD administration reduced appetite, however this effect was lost in the presence of Sitagliptin, suggesting that Abn-CBD may have an effect on PYY secretion. Contrary to this, AM251 only reduced appetite when administered in combination with Sitagliptin. This suggests that AM251 increases GLP-1 and GIP secretion as the inhibitor will allow both hormones to circulate for longer thus having a greater effect. Sitagliptin treatment alone did not reduce appetite as it is likely that GLP-1 secretion was not increased as significantly as when AM251 was present.

In order to observe the long term effects of Abn-CBD in this study, a multiple low dose streptozotocin model of diabetes was utilised as has been used previously (McKillop *et al.* 2016). Streptozotocin is a glucose analogue which is cytotoxic to the insulin secreting β -cells of the pancreas (Szkudelski 2001). Due to the similarities in structure with glucose, streptozotocin is able to enter β -cells through the GLUT2 receptor and induce diabetes through β -cell toxicity and a T-cell mediated immune response against β -cells in the pancreas (Wang, Gleichmann 1998, Szkudelski 2001). The alkylating properties of streptozotocin mean that free radicals are added to DNA present in β -cells and destroying the cell and preventing it from metabolising glucose due to the breakdown of mitochondrial DNA (Szkudelski 2001). The effects of multiple low dose streptozotocin induced diabetes on rats has previously shown to result in mild hyperglycaemia,

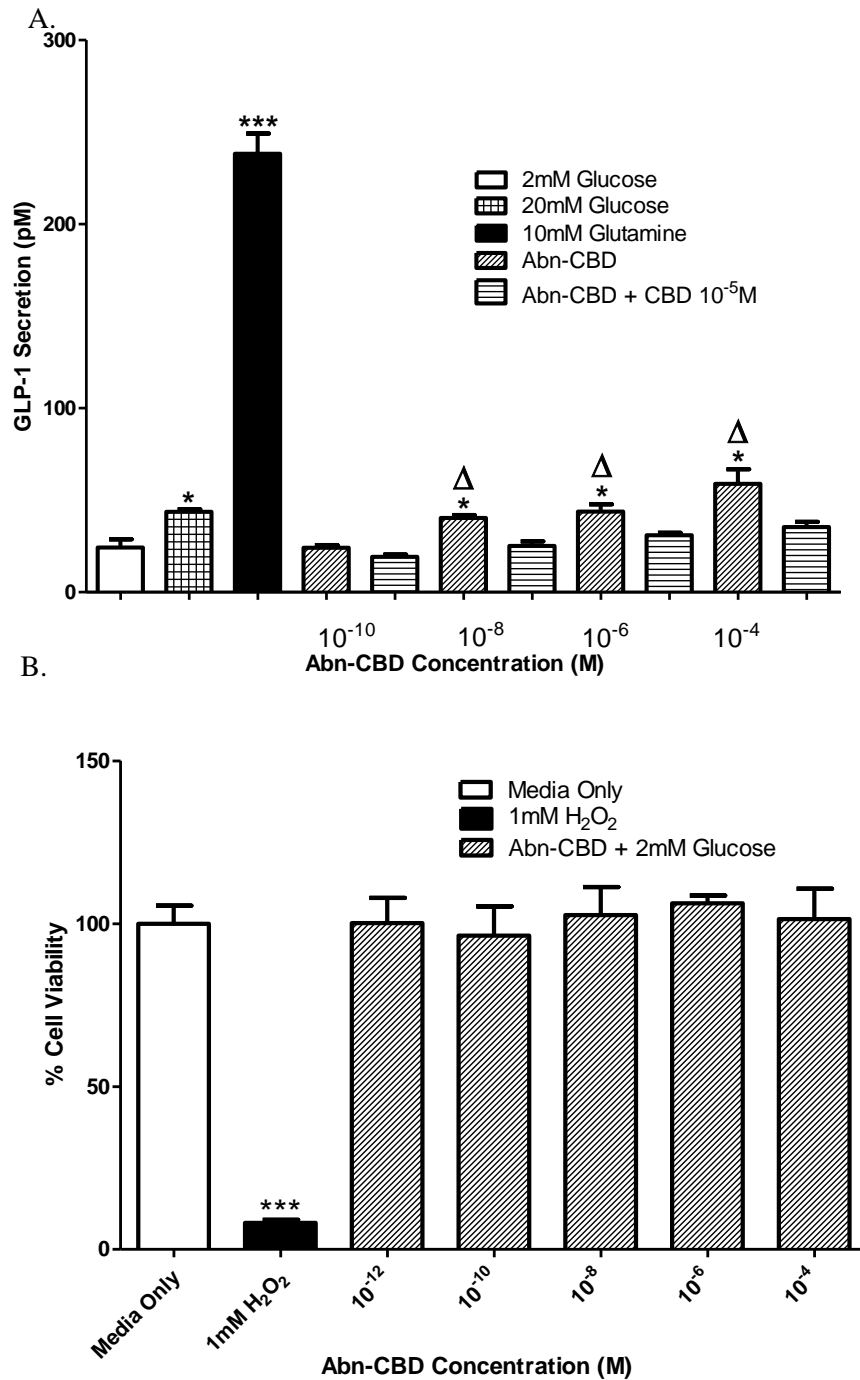
hypoinsulinaemia, hyperphagia and polydipsia with no adverse effects on weight gain (Kobayashi, Olefsky 1979).

Treatment of mice with streptozotocin led to increased non-fasting hyperglycaemia, food intake, fluid intake and GIP secretion while plasma insulin and the ability to control glycaemia after glucose intake was reduced due to reduced insulin sensitivity and lower insulin secretion in response to glucose. Treatment of streptozotocin induced diabetic mice with Abn-CBD reduced LDL cholestetrol, non-fasting hyperglycaemia, body fat, hyperglycaemia after glucose challenge, fluid intake and food intake. Daily oral Abn-CBD treatment increased incretin hormone and insulin secretion, insulin response to glucose, insulin sensitivity and bone mineral density in the femur. Abn-CBD combined with Sitagliptin reduced body fat, non-fasting hyperglycaemia, hyperglycaemia in response to glucose challenge, food intake and fluid intake. Plasma insulin, insulin secretion in response to glucose, incretin hormone secretion and insulin sensitivity were all increased. Previous work has shown that Abn-CBD administration improved glucose homeostasis (McKillop *et al.* 2016), however this is the first study to show the long term effects of this agonist on incretin hormone secretion and is the first study to demonstrate improved glycaemic control in combination with Sitagliptin. In order to better elucidate the effects seen in this study, further studies in GPR55 knockout and other diabetic animal models are required.

This study differed from previous work as it looked at the direct action of GPR55 activation on incretin hormones as well as being the first study to use Abn-CBD in combination with the DPP-IV inhibitor Sitagliptin. Daily oral administration of both Abn-CBD and Abn-CBD in combination with Sitagliptin increased insulin secretion in non-fasted mice while also increasing circulating incretin hormone levels. These factors all lead to reduced blood glucose in both treatment groups. There is currently only one published study demonstrating that GPR55 activation can increase GLP-1 secretion directly (Harada *et al.* 2017) and this was done using LPI. Therefore this is the first study to show direct action of Abn-CBD and AM251 can increase incretin hormone secretion to better help regulate glucose homeostasis.

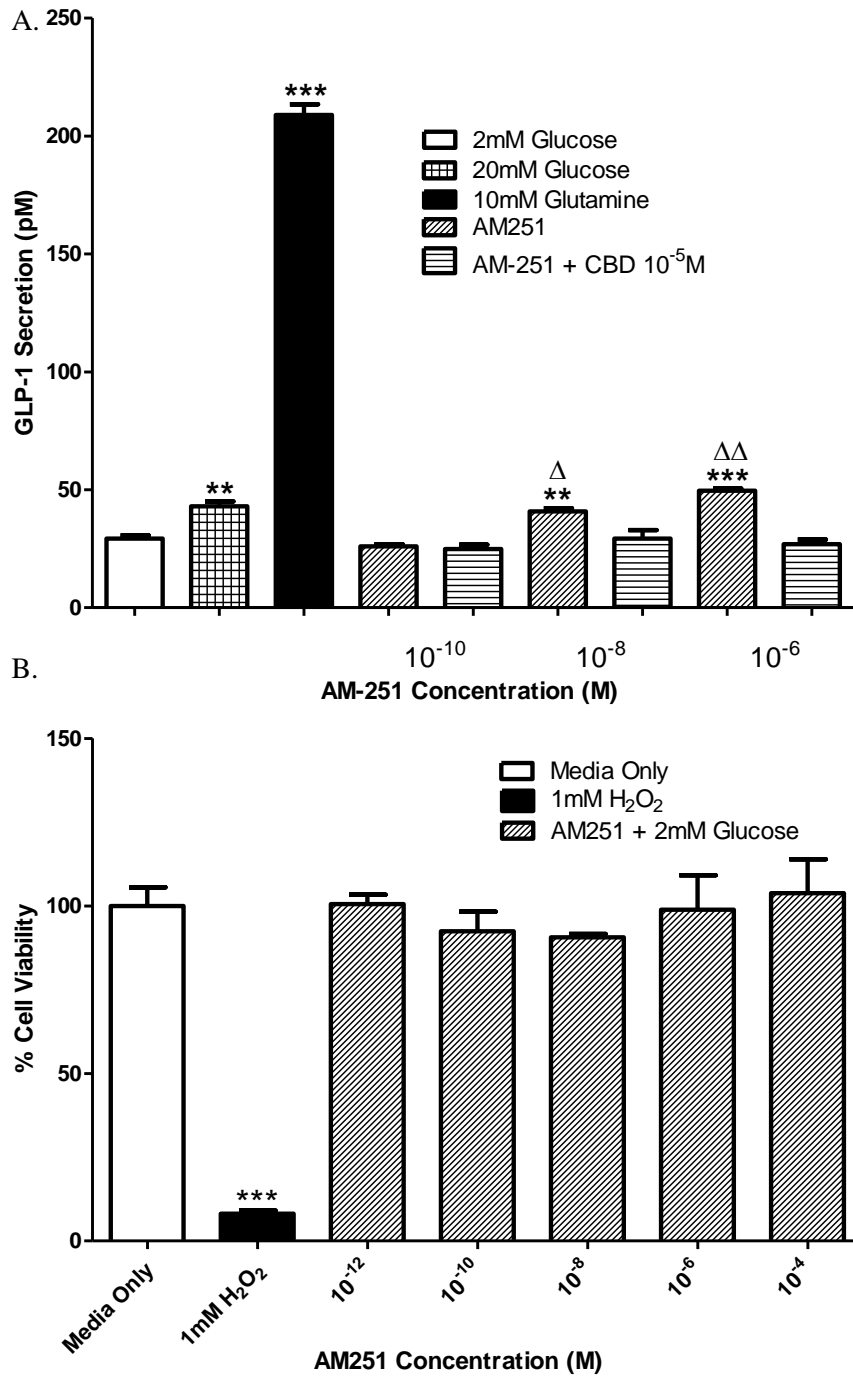
In conclusion, the research described in this study confirms the insulinitropic effect of GPR55 agonists in β -cells of the pancreas both *in vitro* and *in vivo*. GPR55 agonists also demonstrated an ability to increase GLP-1 secretion *in vitro* while only those agonists which work through other GPCRs were able to increase GIP secretion. The effects of these agonists on GLP-1 secretion were confirmed with qPCR demonstrating that GPR55 agonists increase proglucagon gene expression. Oral administration of GPR55 agonist Abn-CBD and AM251 resulted in a significant glucose lowering and insulinitropic effect *in vivo* and when administered in combination with a DPP-IV inhibitor. Activation of GPR55 has potential as a novel therapy in metabolic disorders such as Type 2 diabetes.

Figure 6.1: Effects of Abn-CBD on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



Effect of GPR55 agonist Abn-CBD (10⁻¹²-10⁻⁴M) and GPR55 antagonist CBD (10⁻⁵M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * p<0.05 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ p<0.05 compared to Abn-CBD treatment alone.

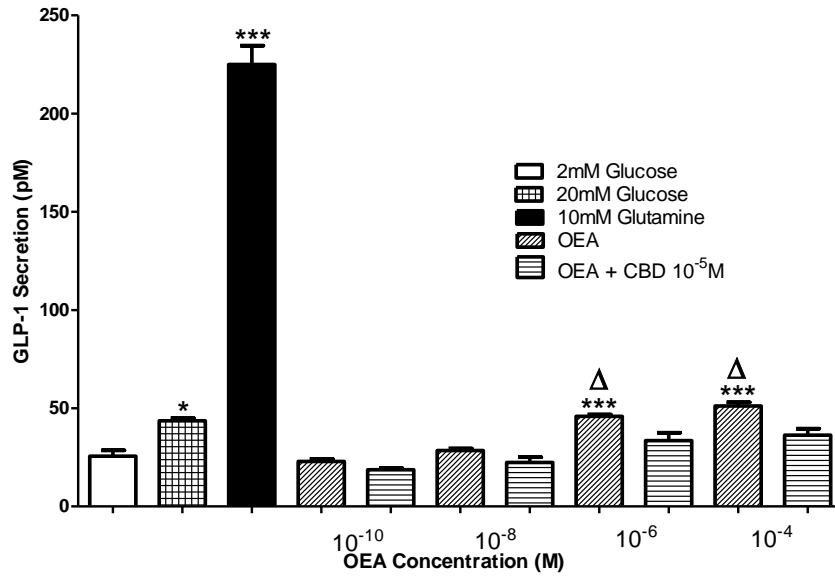
Figure 6.2: Effects of AM251 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



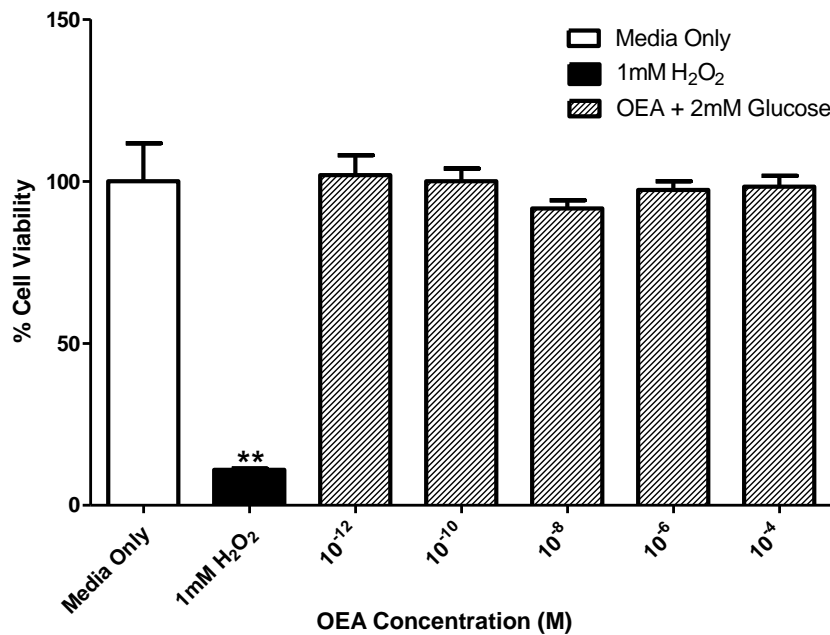
Effect of GPR55 agonist AM251 (10⁻¹²-10⁻⁴M) and GPR55 antagonist CBD (10⁻⁵M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. ** p<0.01 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. ΔΔ p<0.01, ΔΔΔ p<0.001 compared to AM251 treatment alone.

Figure 6.3: Effects of OEA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose

A.

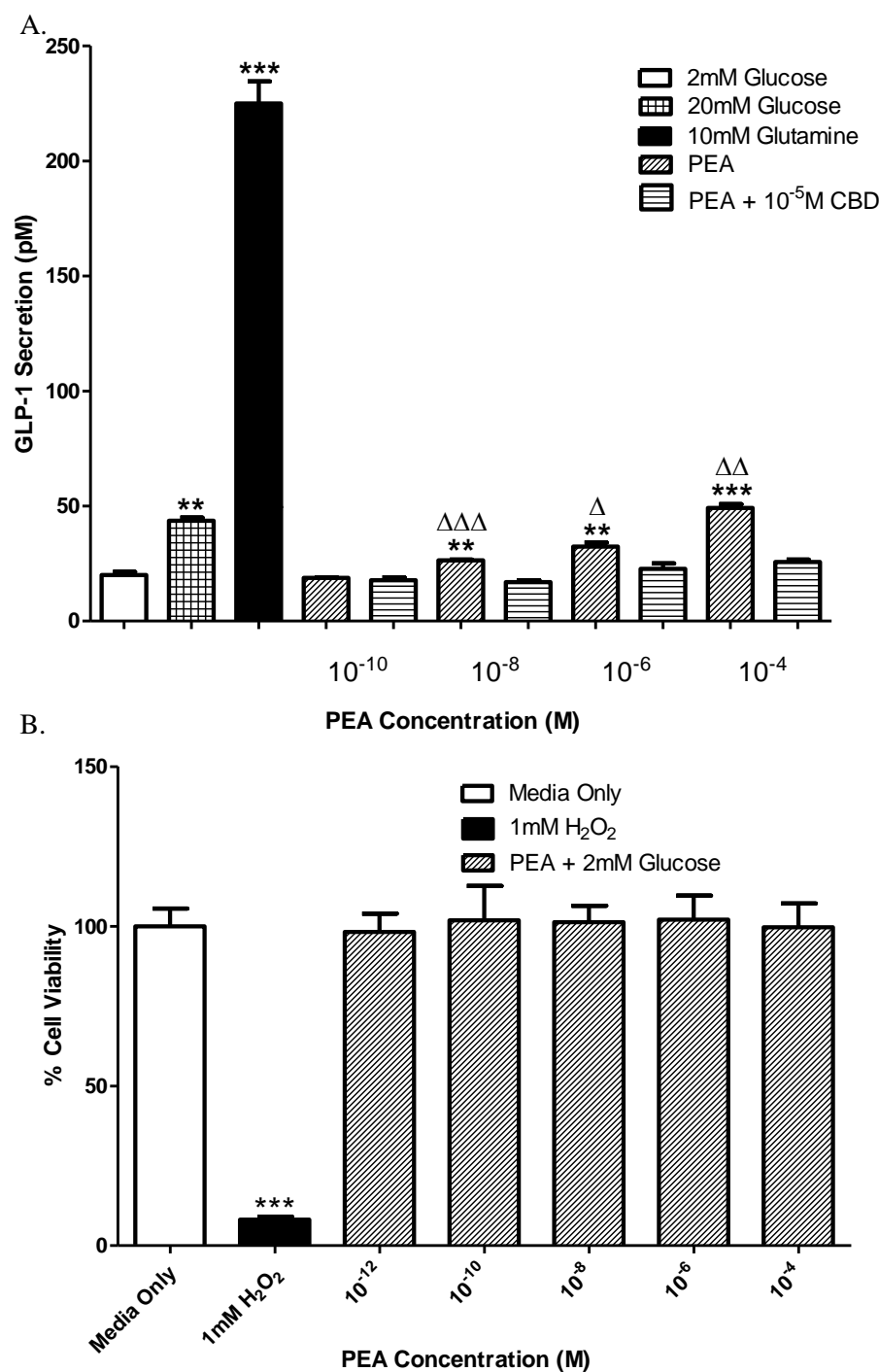


B.



Effect of GPR55 agonist OEA (10^{-12} - 10^{-4} M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ $p < 0.05$ compared to OEA treatment alone.

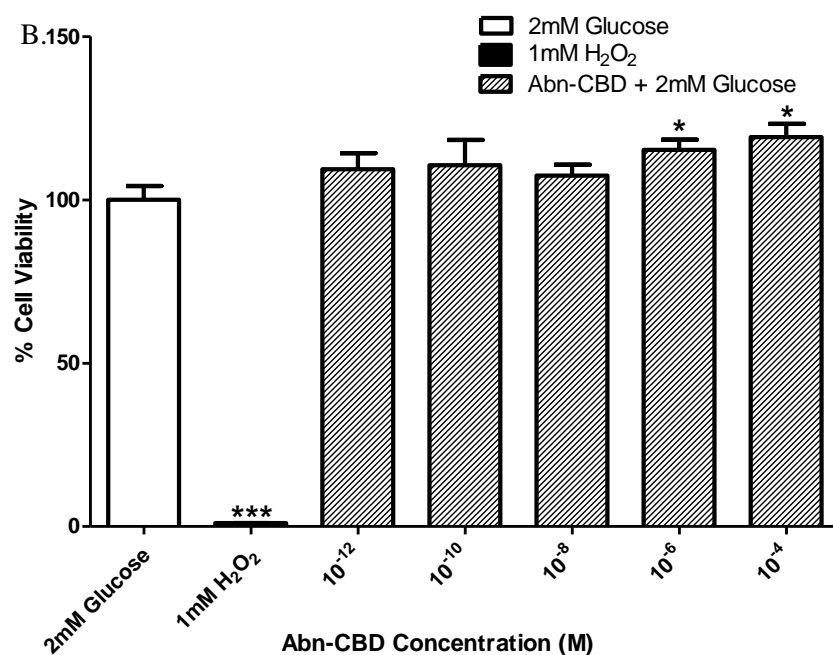
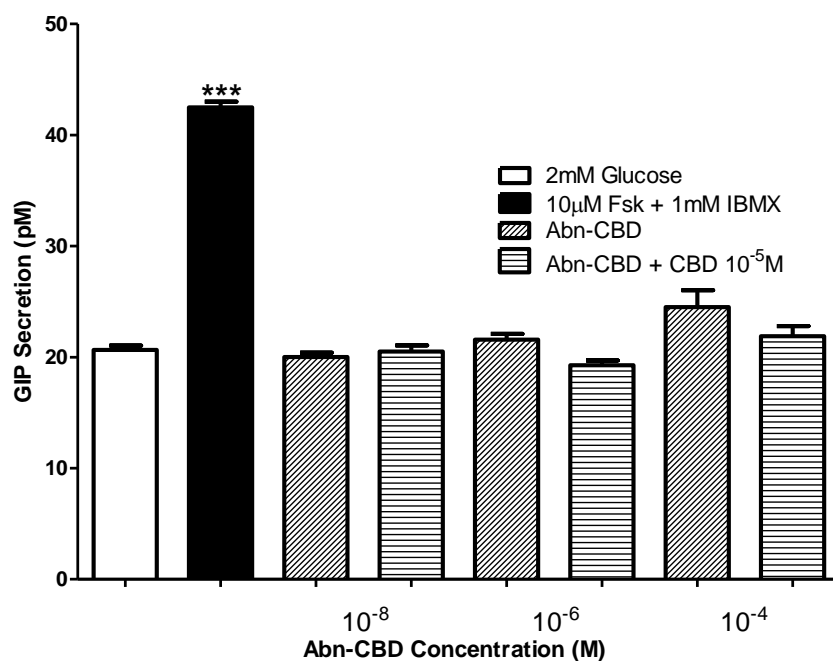
Figure 6.4: Effects of PEA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



Effect of GPR55 agonist PEA (10⁻¹²-10⁻⁴M) and GPR55 antagonist CBD (10⁻⁵M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * p<0.05, ** p<0.01 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ p<0.05 compared to PEA treatment alone.

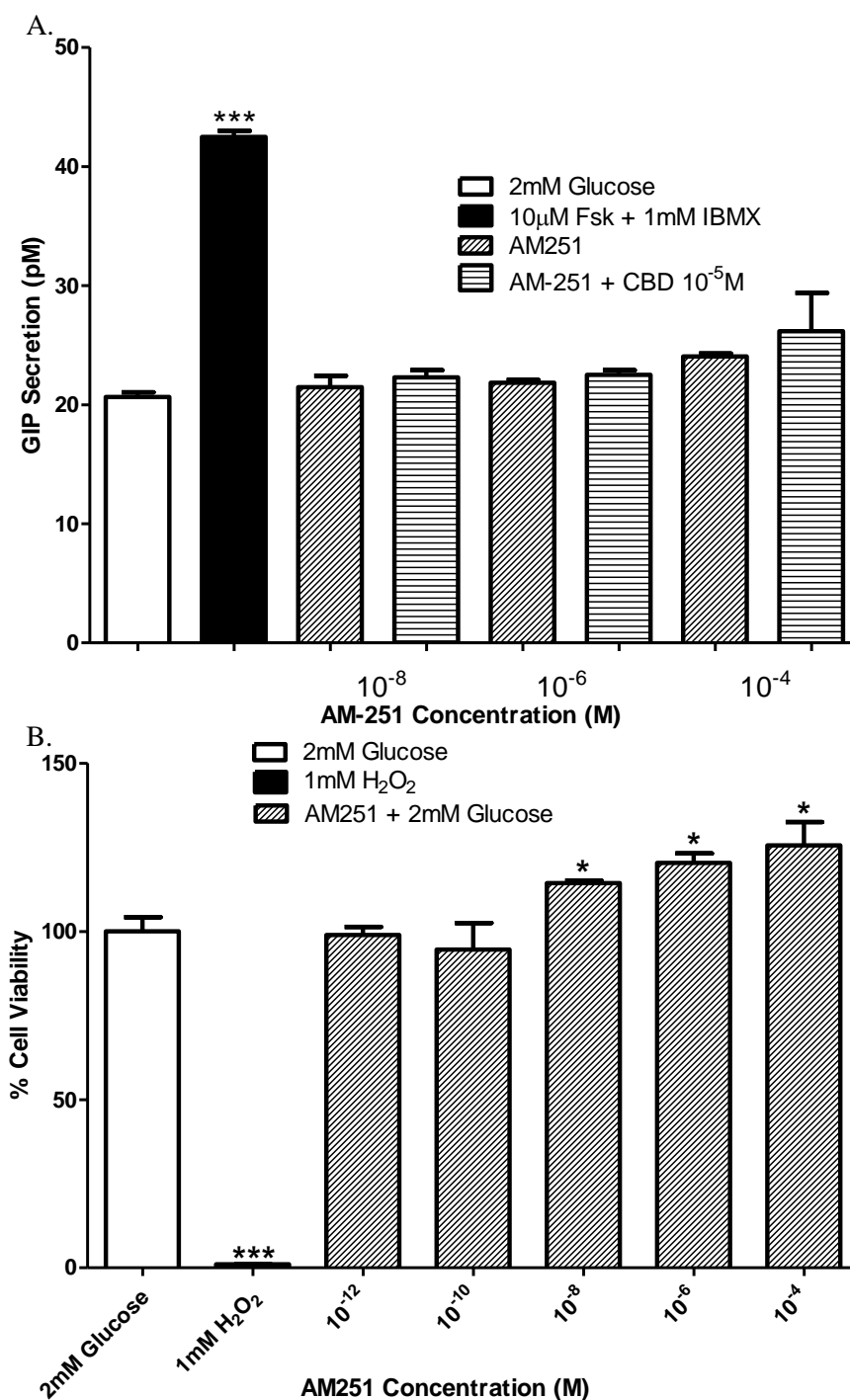
Figure 6.5: Effects of Abn-CBD on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.



Effect of GPR55 agonist Abn-CBD (10^{-12} - 10^{-4} M) and GPR55 antagonist CBD on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM ($n=3$) for GIP secretion and cell viability. * $p<0.05$, and *** $p<0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

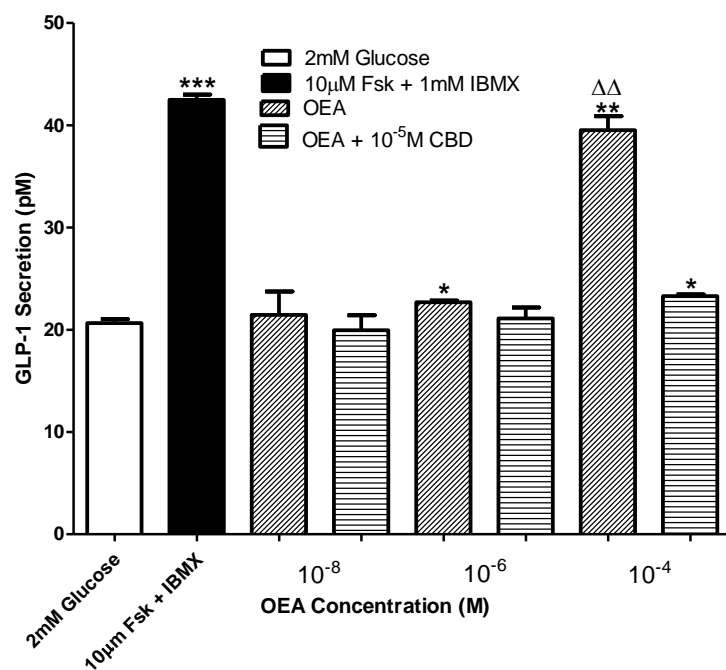
Figure 6.6: Effects of AM251 on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



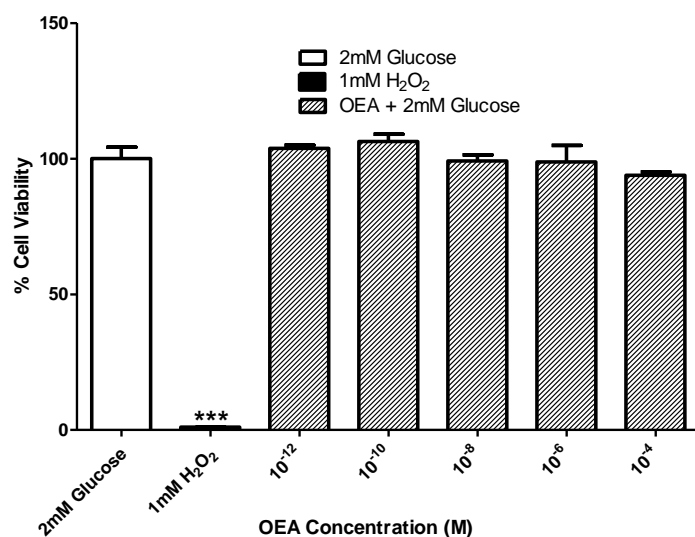
Effect of GPR55 agonist AM251 (10^{-12} - 10^{-4} M) and GPR55 antagonist CBD on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 6.7: Effects of OEA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose

A.

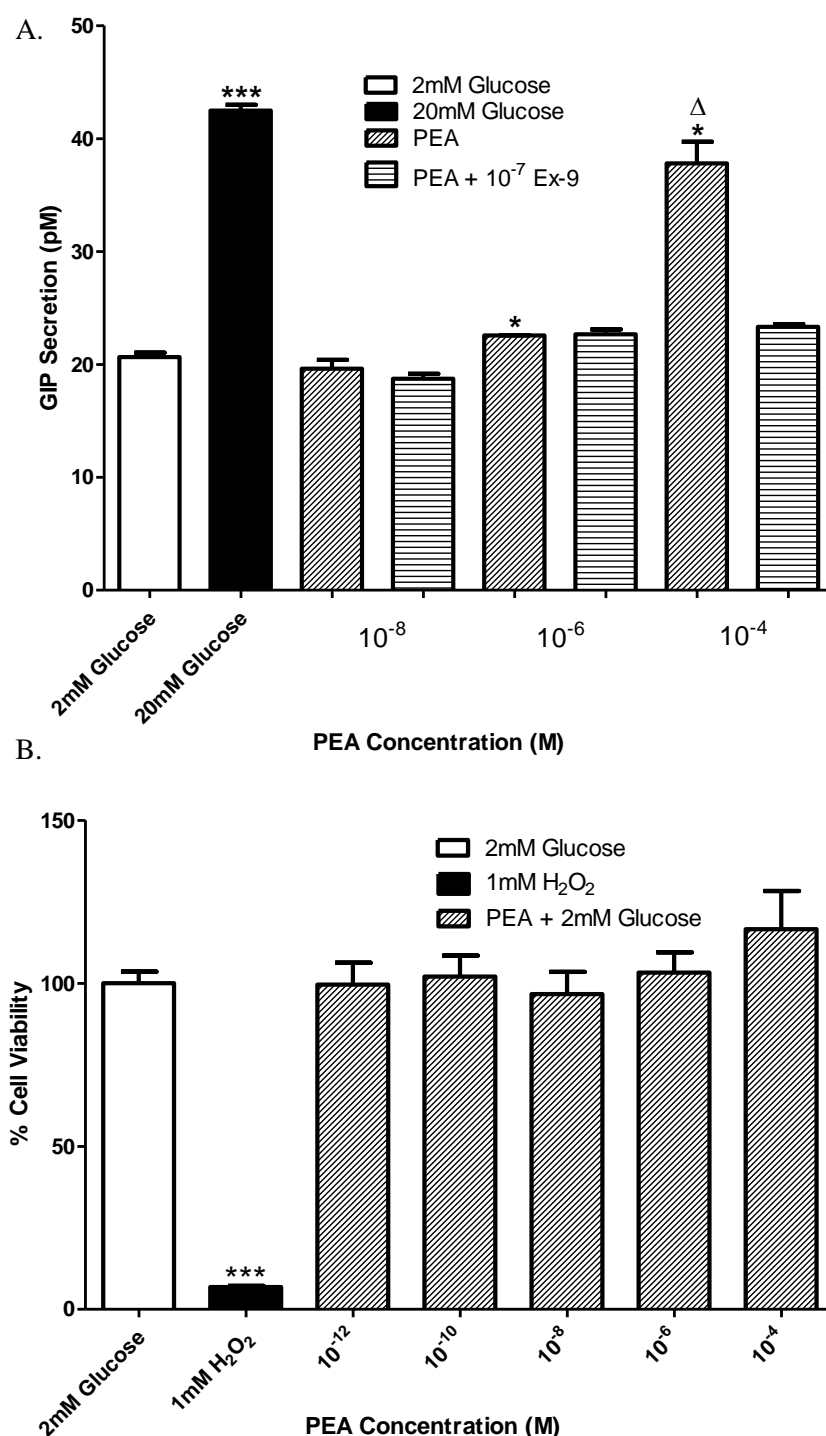


B.



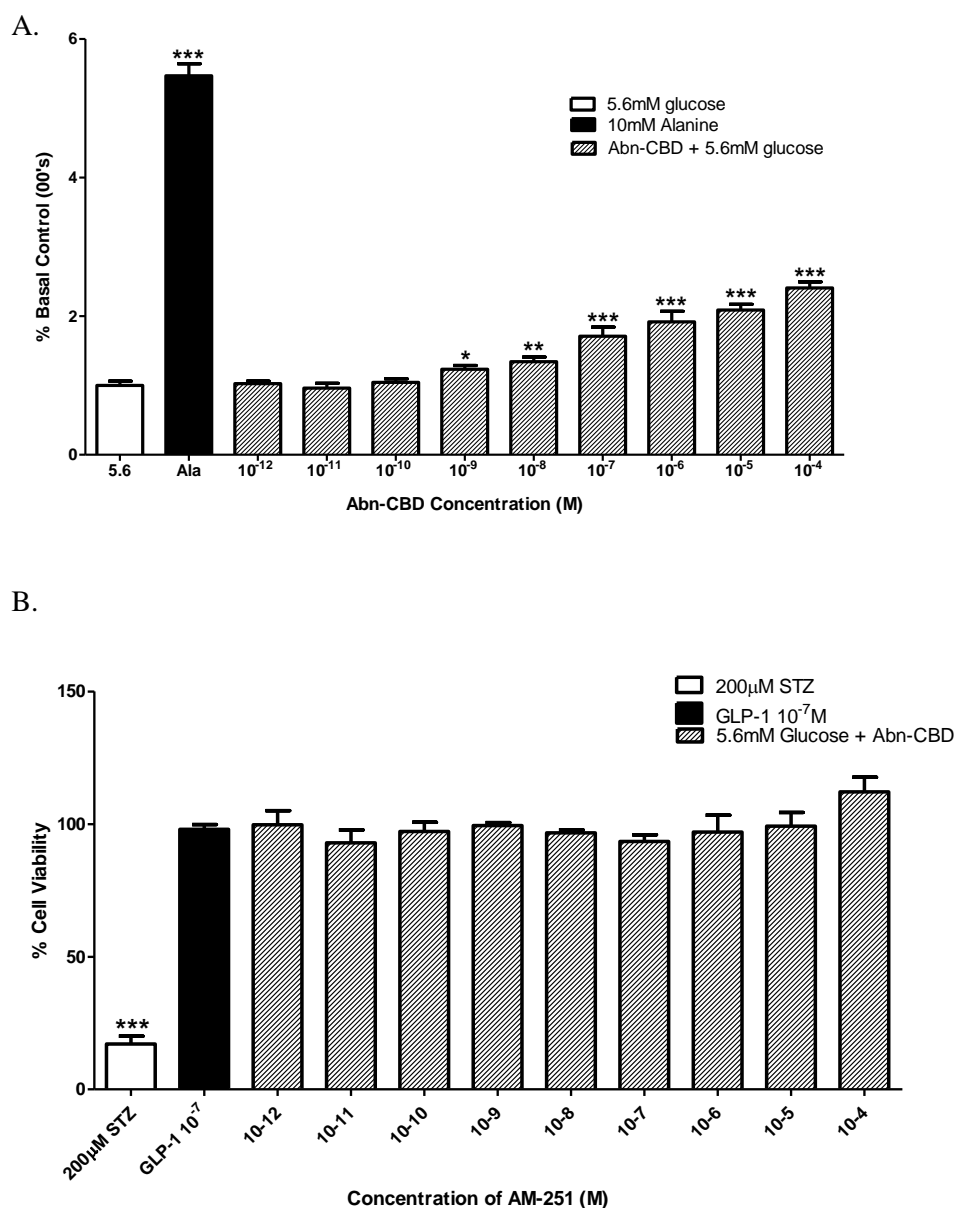
Effect of GPR55 agonist OEA (10^{-12} - 10^{-4} M) and GPR55 antagonist CBD on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 6.8: Effects of PEA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



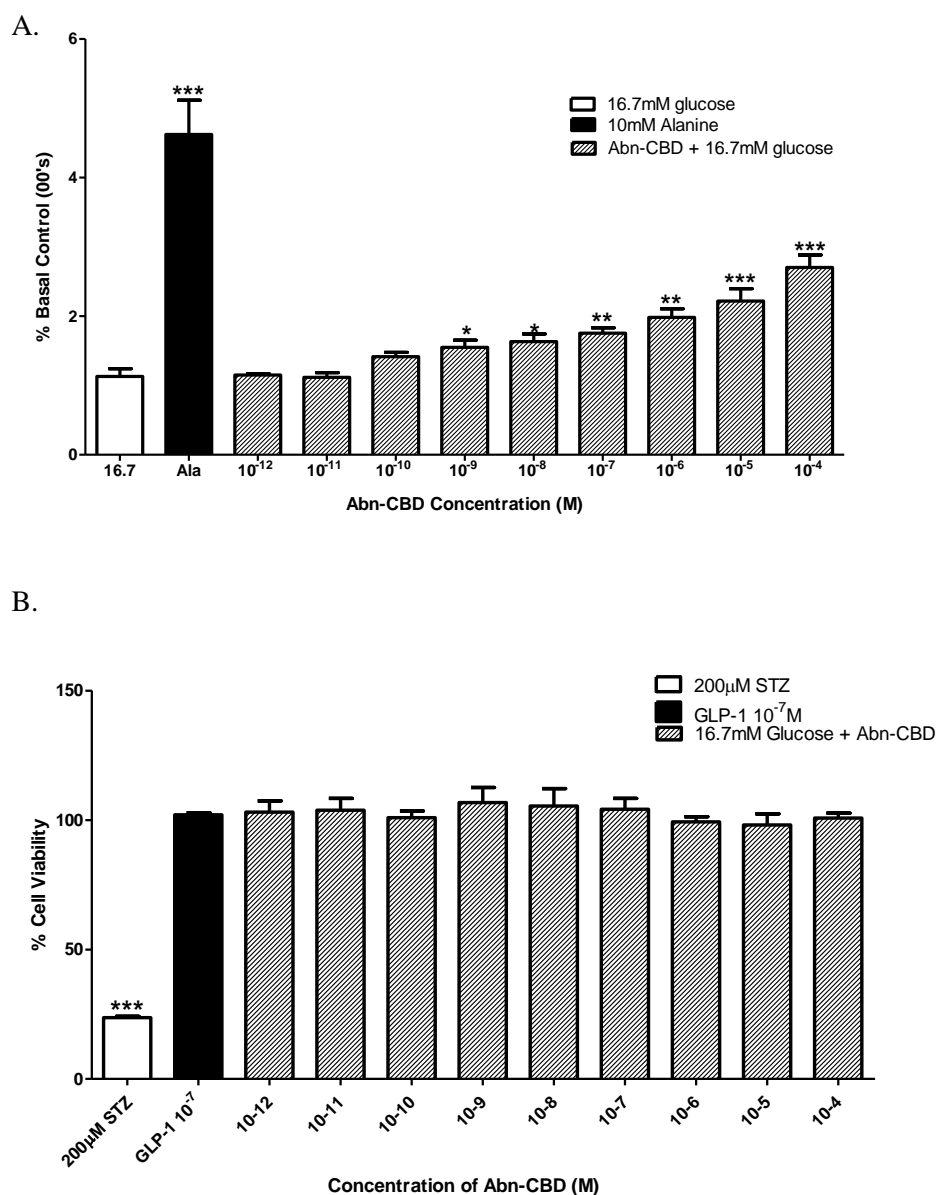
Effect of GPR55 agonist PEA (10^{-12} - 10^{-4} M) and GPR55 antagonist CBD on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 6.9: Effects of Abn-CBD on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose



Effect of Abn-CBD (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

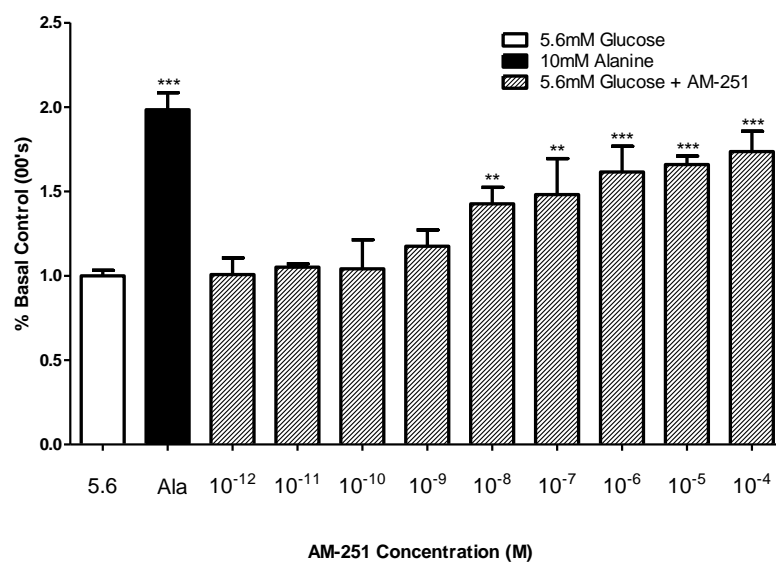
Figure 6.10: Effects of Abn-CBD on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



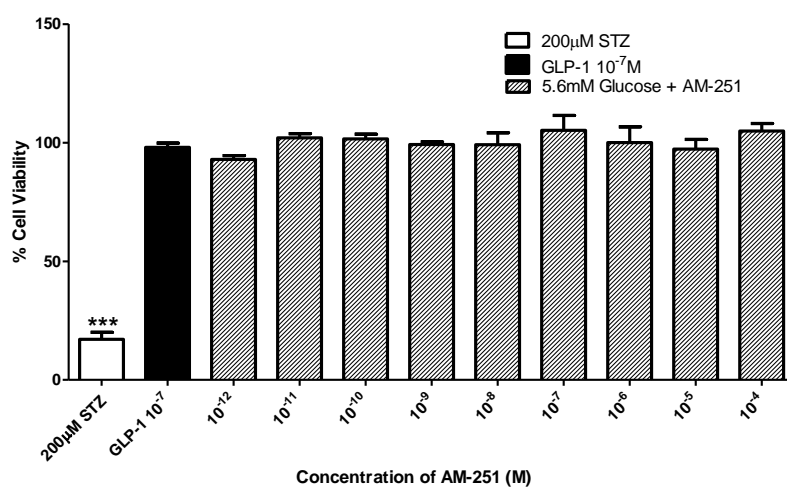
Effect of Abn-CBD (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 6.11: Effects of AM251 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.

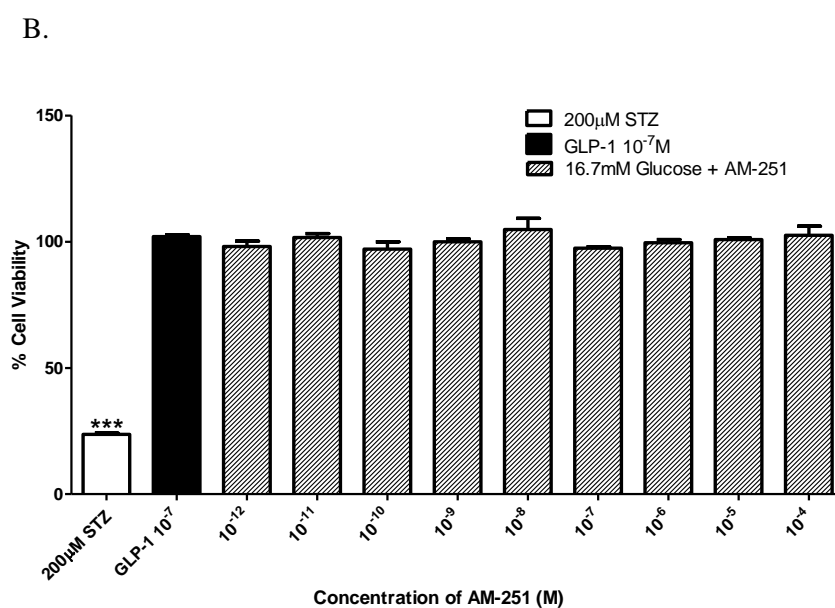
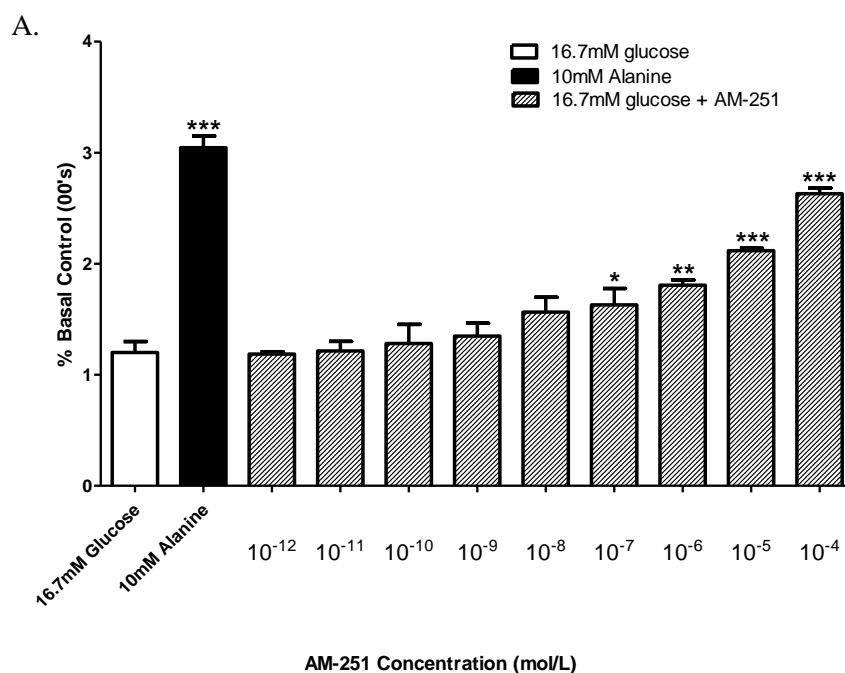


B.



Effect of AM251 (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability.* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

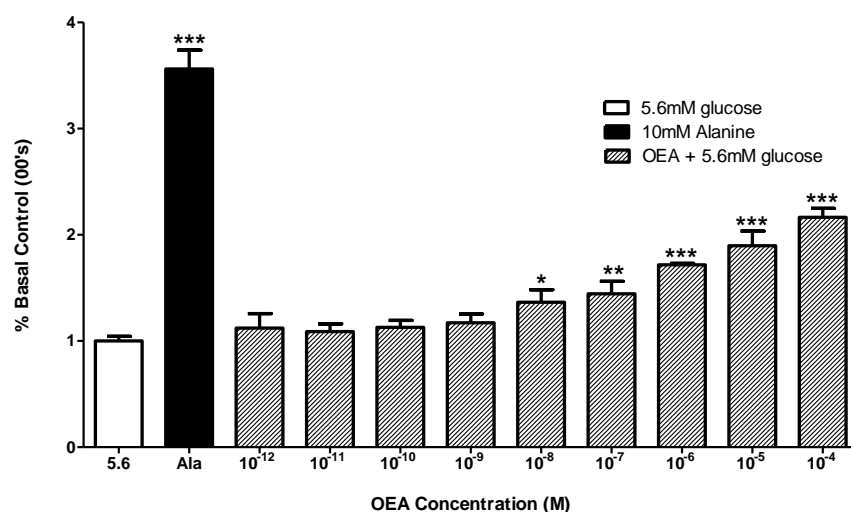
Figure 6.12: Effects of AM251 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



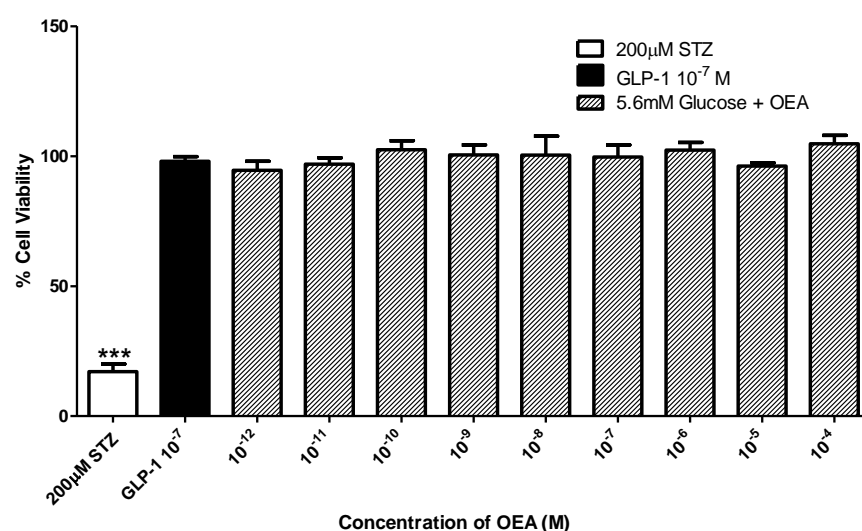
Effect of AM251 (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 6.13: Effects of OEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.

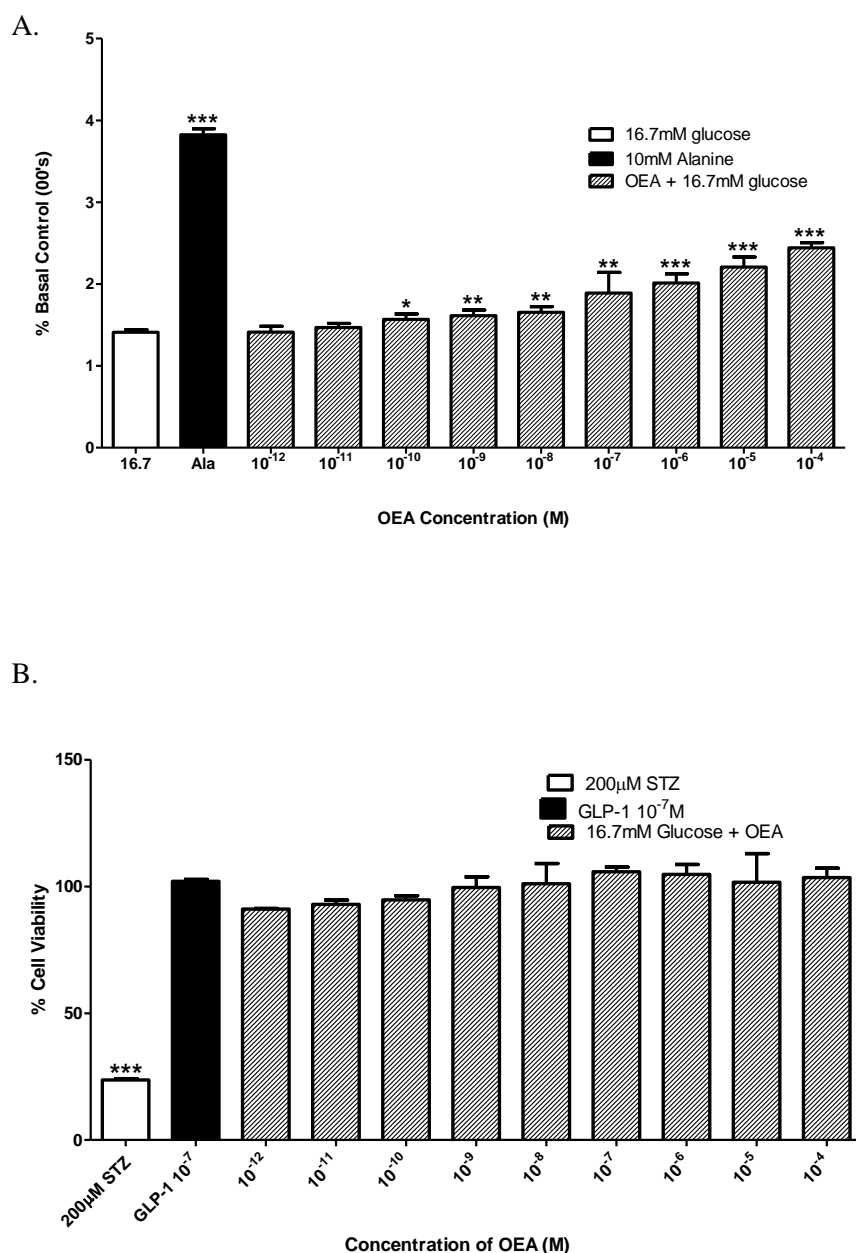


B.



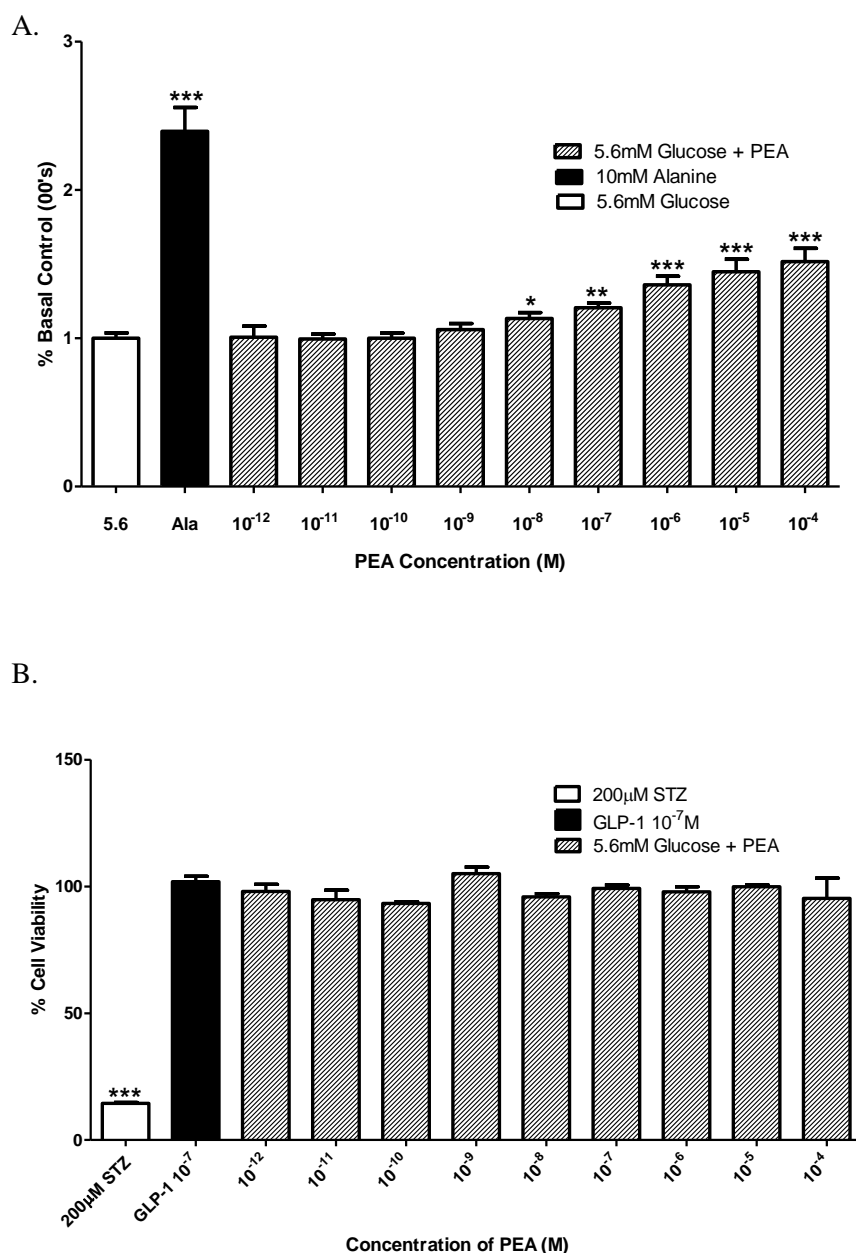
Effect of OEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 6.14: Effects of OEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



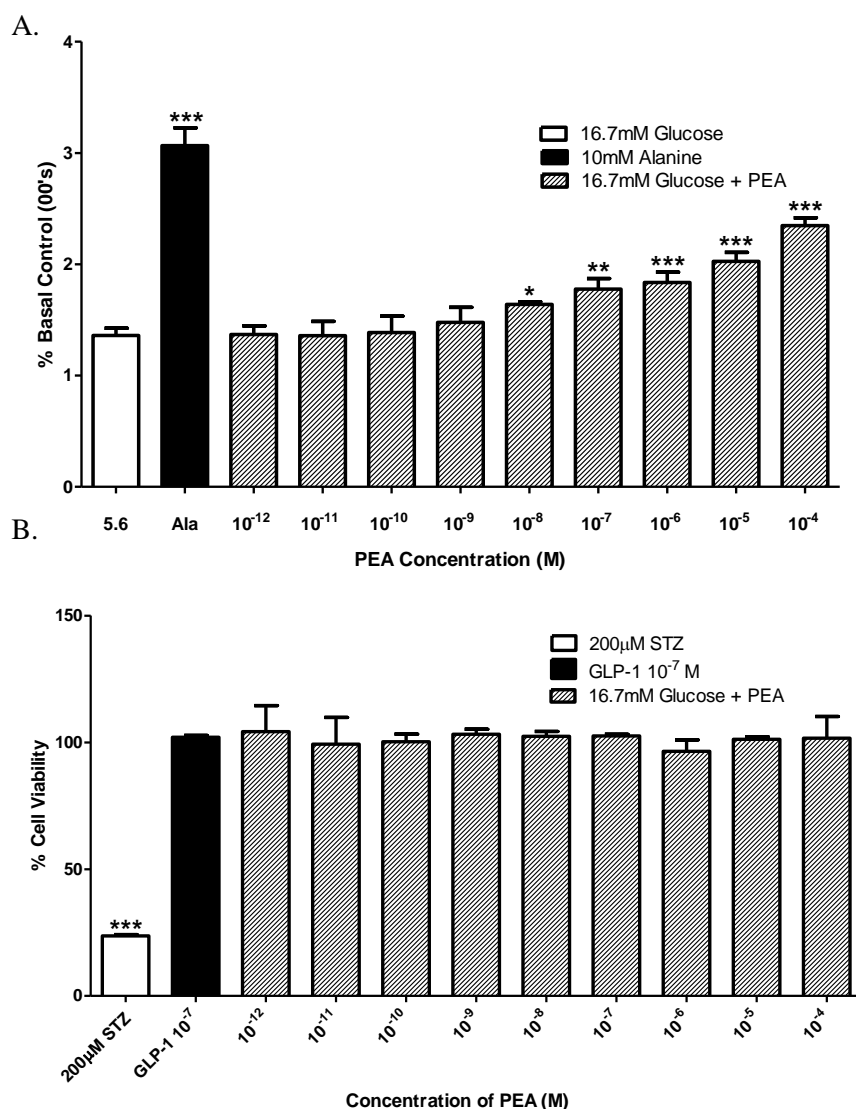
Effect of OEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 6.15: Effects of PEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose



Effect of PEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

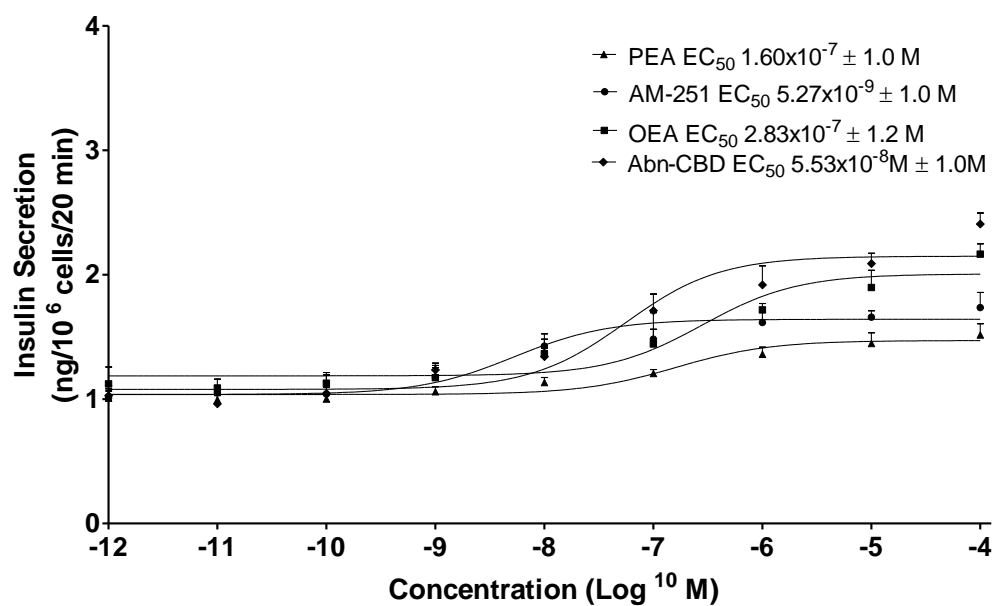
Figure 6.16: Effects of PEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



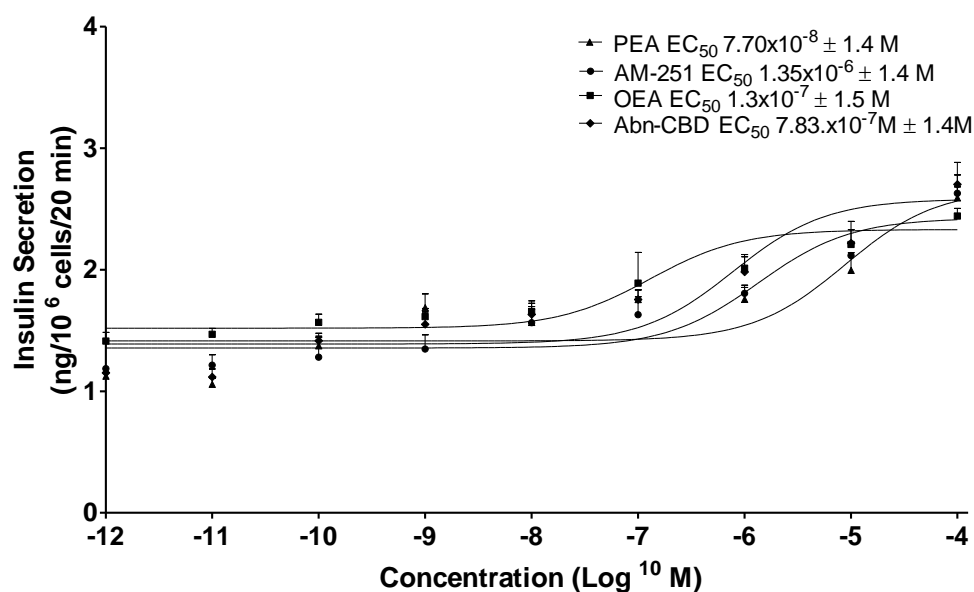
Effect of PEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 6.17: EC₅₀ of GPR44 agonists Abn-CBD, AM251, OEA and PEA in BRIN-BD11 cells

A.

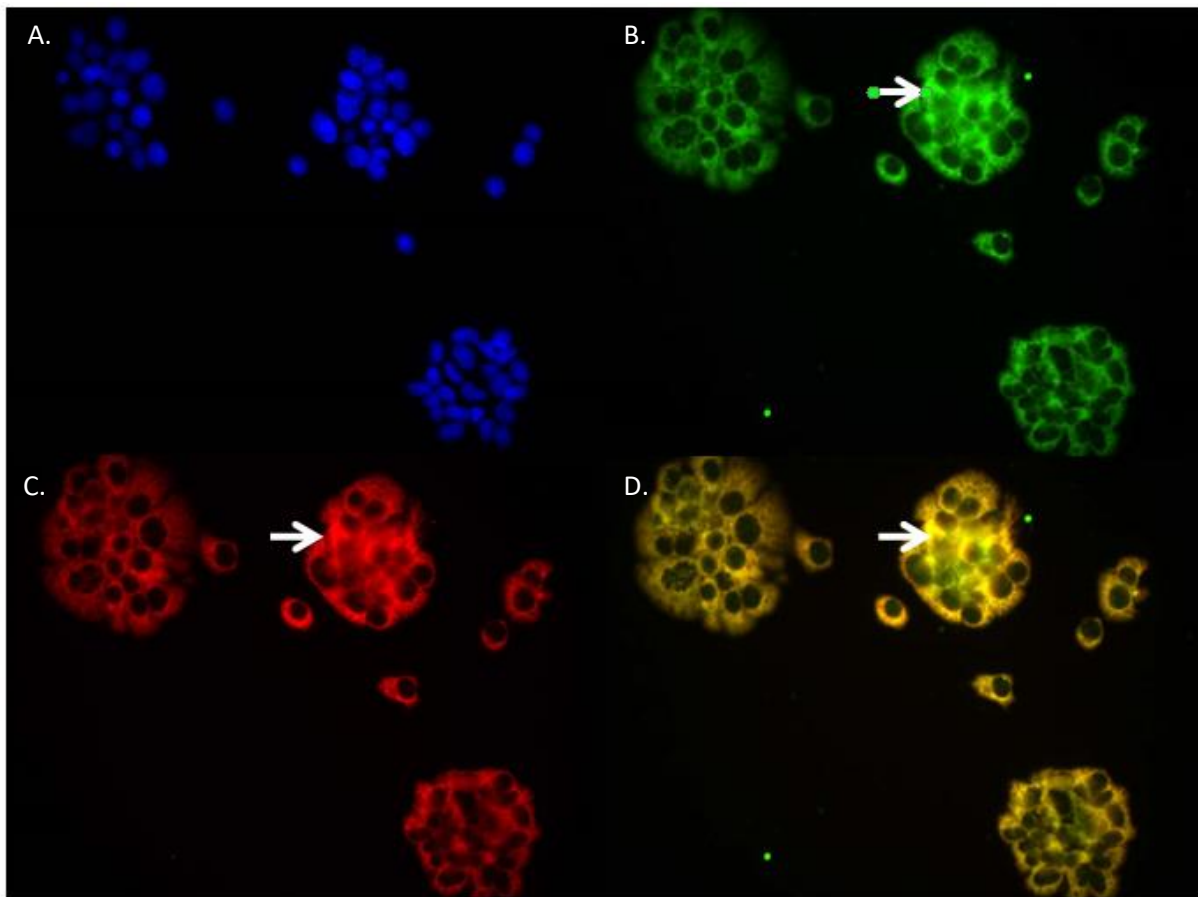


B.



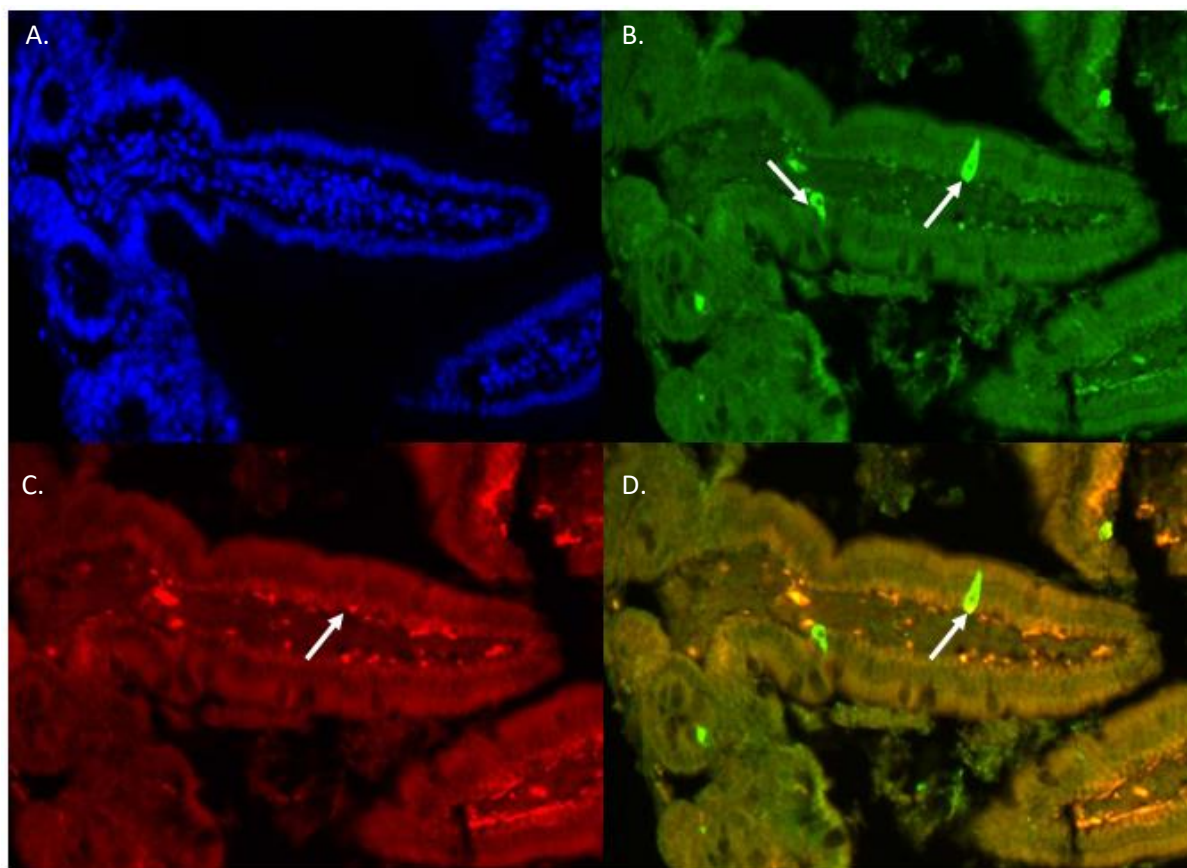
EC₅₀ values for GPR55 agonists Abn-CBD, AM251, OEA and PEA in BRIN-BD11 cells in 5.6mM glucose (A) and 16.7mM glucose (B).

Figure 6.18: Immunofluorescence staining of GLP-1 and GPR55 in intestinal GLUTag cells



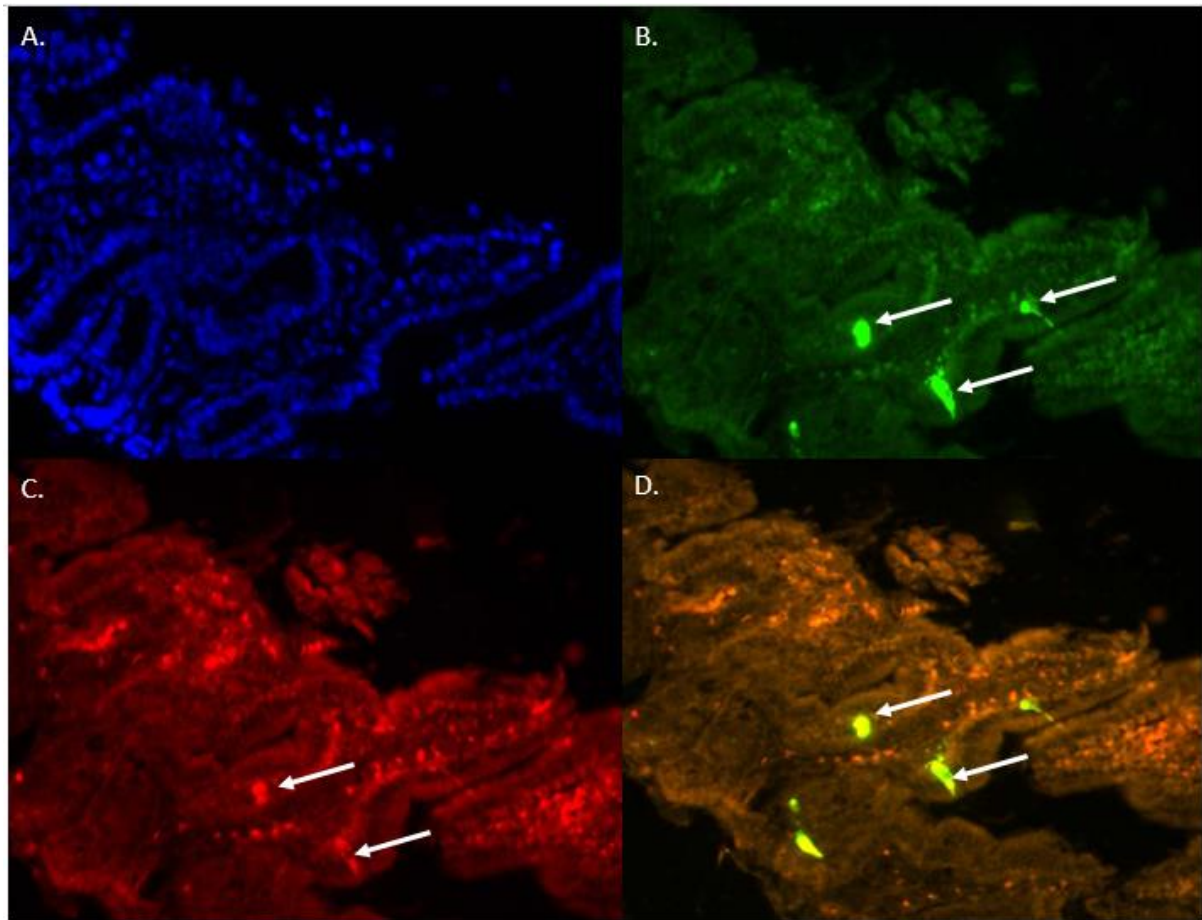
Distribution of (A) DAPI, (B) GLP-1, (C) GPR55 and (D) combined fluorescence of GLP-1 and GPR55 in GLUTag cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.19: Immunofluorescence staining of GLP-1 and GPR55 in lean NIH Swiss mouse small intestine



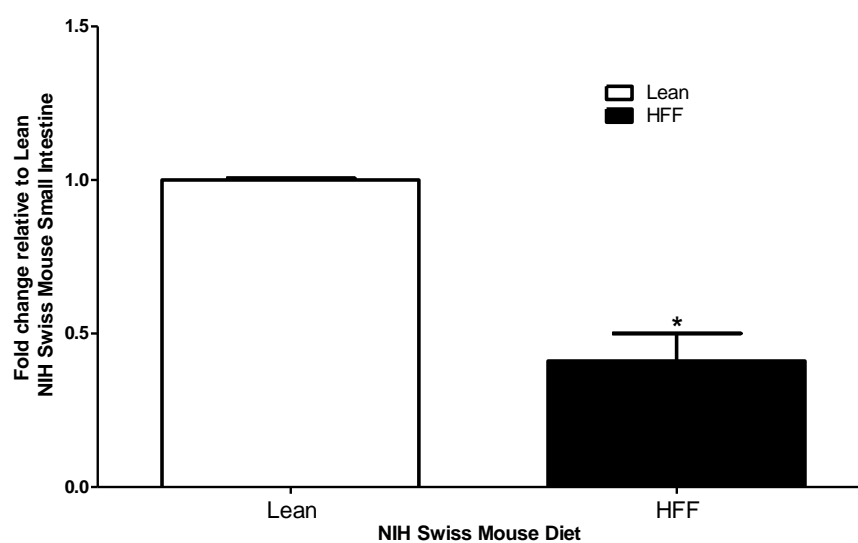
Distribution of (A) DAPI, (B) GLP-1, (C) GPR55 and (D) combined fluorescence of GLP-1 and GPR55 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.20: Immunofluorescence staining of GLP-1 and GPR55 in HFF NIH Swiss mouse small intestine



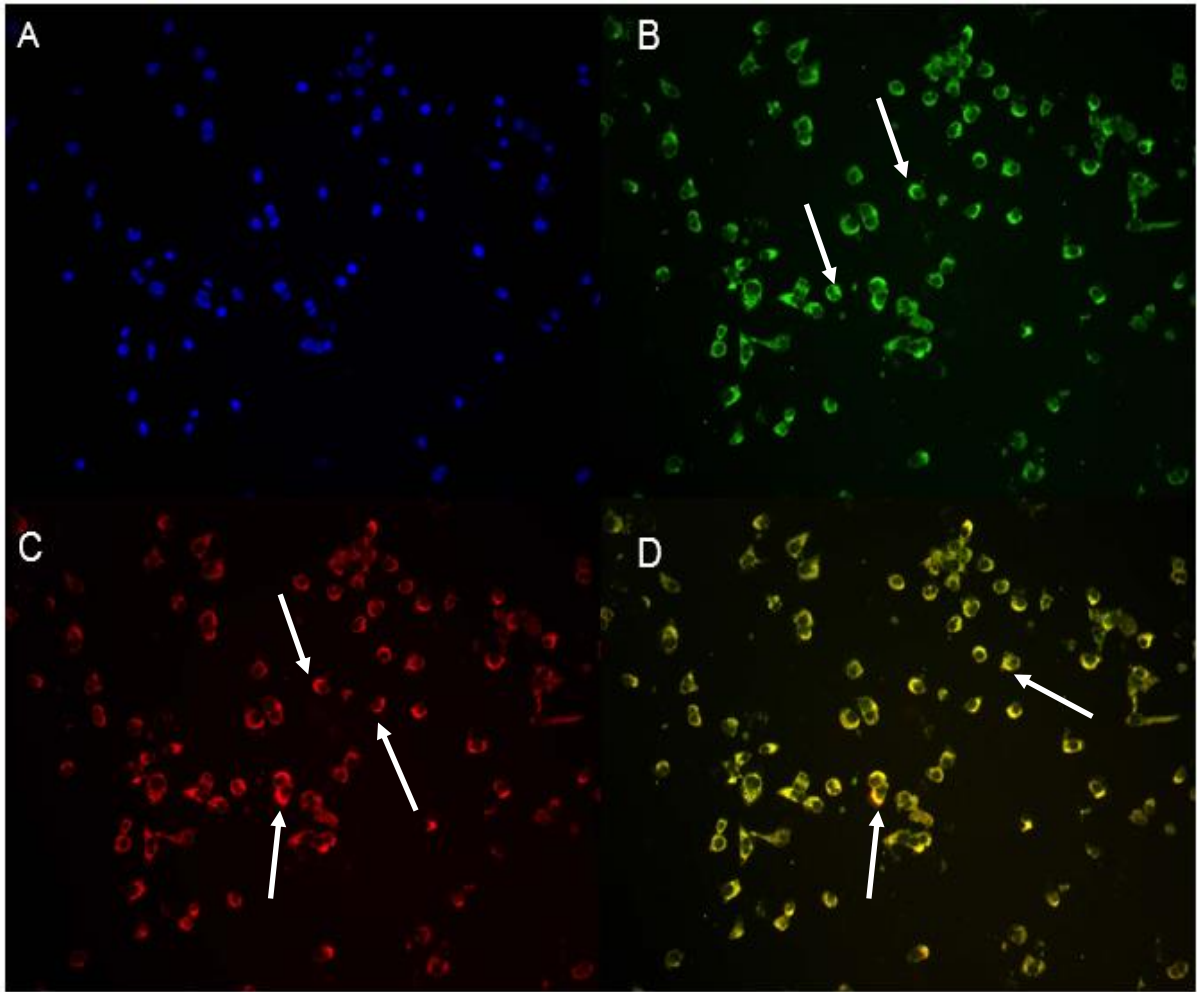
Distribution of (A) DAPI, (B) GLP-1, (C) GPR55 and (D) combined fluorescence of GLP-1 and GPR55 in HFF NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.21: GPR55 gene expression in lean and HFF NIH Swiss mouse small intestine



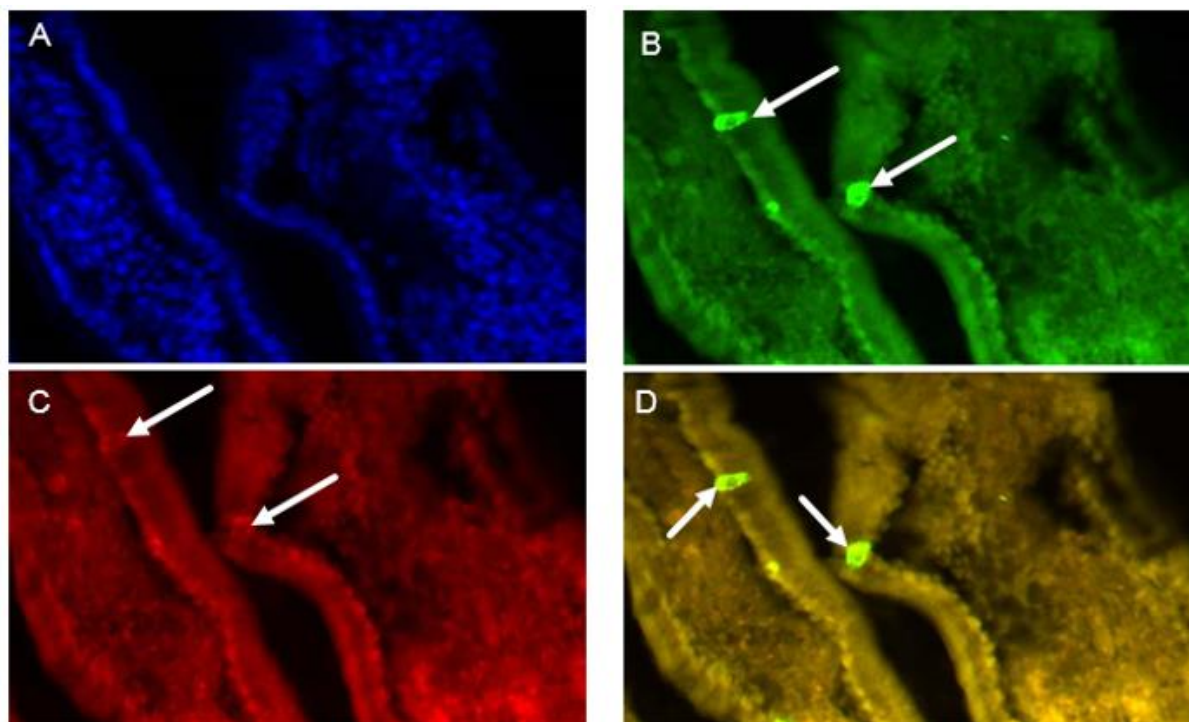
Effect of HFF diet on GPR55 gene expression in male NIH Swiss mouse small intestine. * $p < 0.05$ compared to lean mice (n=6).

Figure 6.22: Immunofluorescence staining of GIP and GPR55 in pGIPneo STC-1 cells



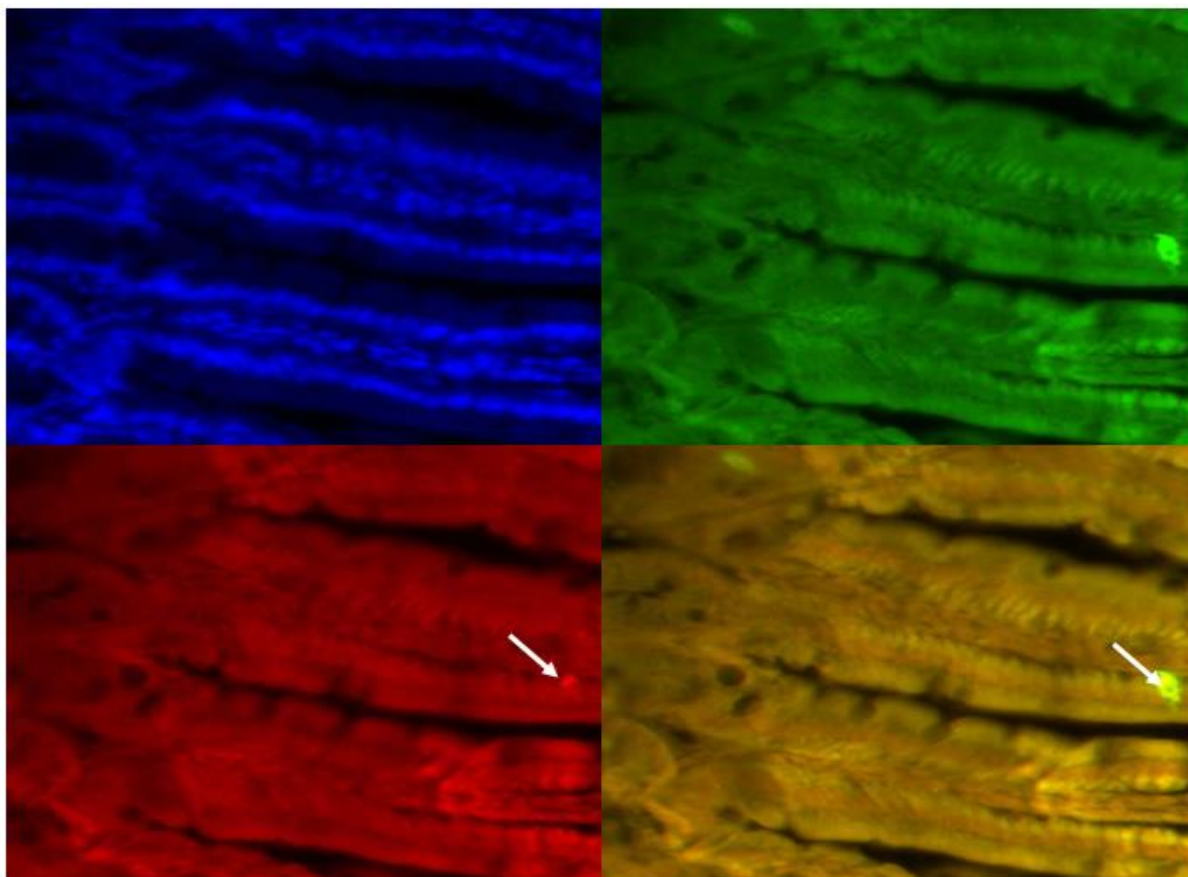
Distribution of (A) DAPI, (B) GIP, (C) GPR55 and (D) combined fluorescence of GIP and GPR55 in pGIPneo STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.23: Immunofluorescence staining of GIP and GPR55 in lean NIH Swiss mouse small intestine



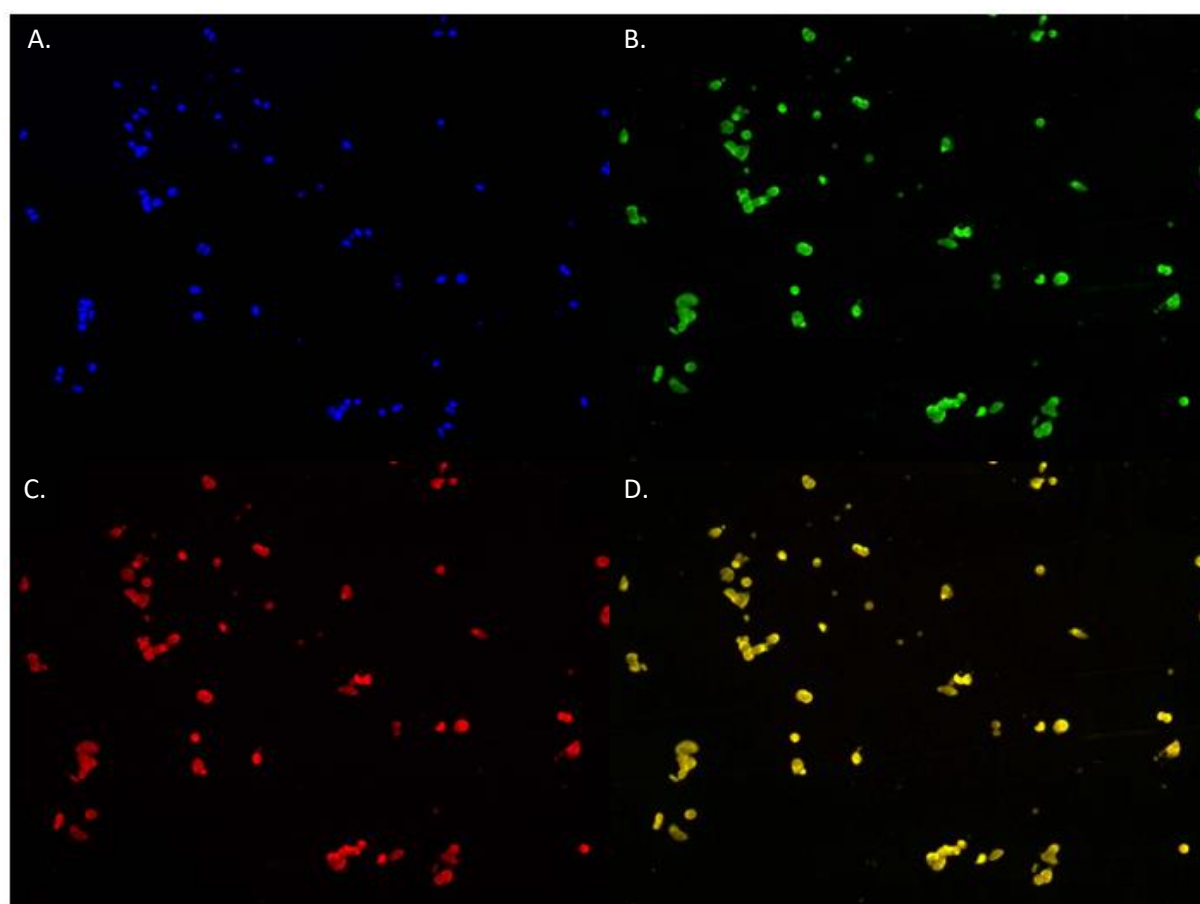
Distribution of (A) DAPI, (B) GIP, (C) GPR55 and (D) combined fluorescence of GIP and GPR55 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.24: Immunofluorescence staining of GIP and GPR55 in HFF NIH Swiss mouse small intestine



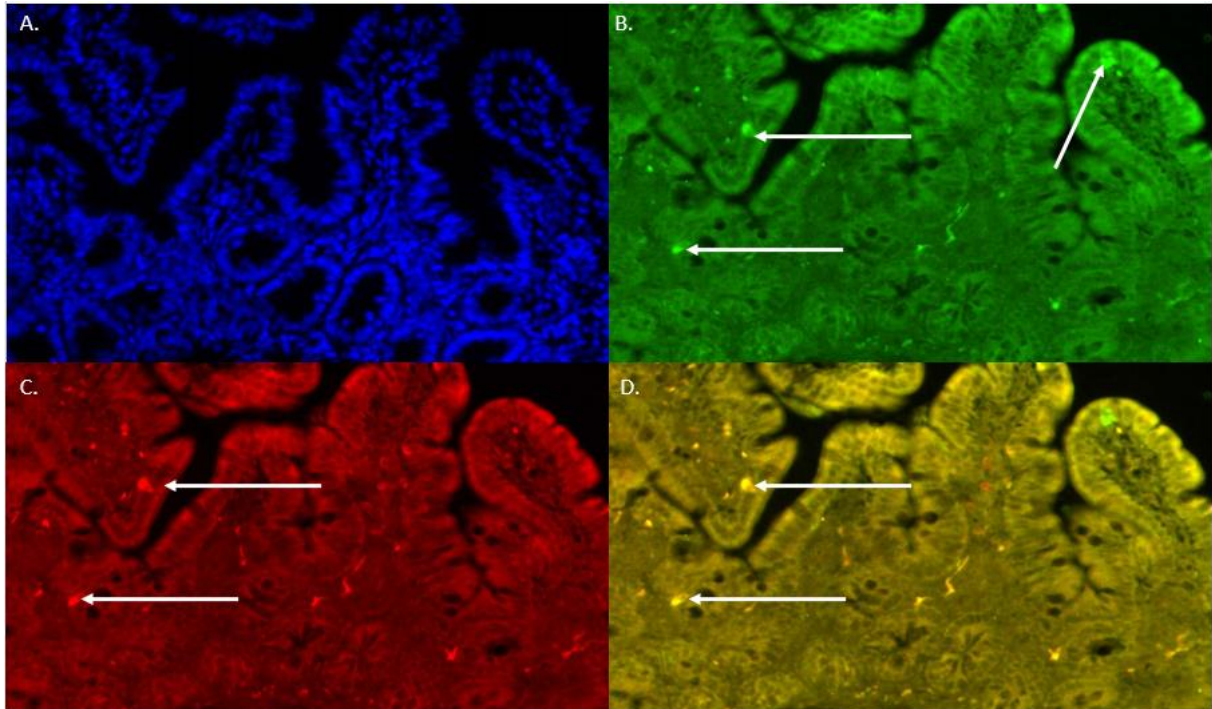
Distribution of (A) DAPI, (B) GIP, (C) GPR55 and (D) combined fluorescence of GIP and GPR55 in HFF NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.25: Immunofluorescence staining of PYY and GPR55 in STC-1 cells



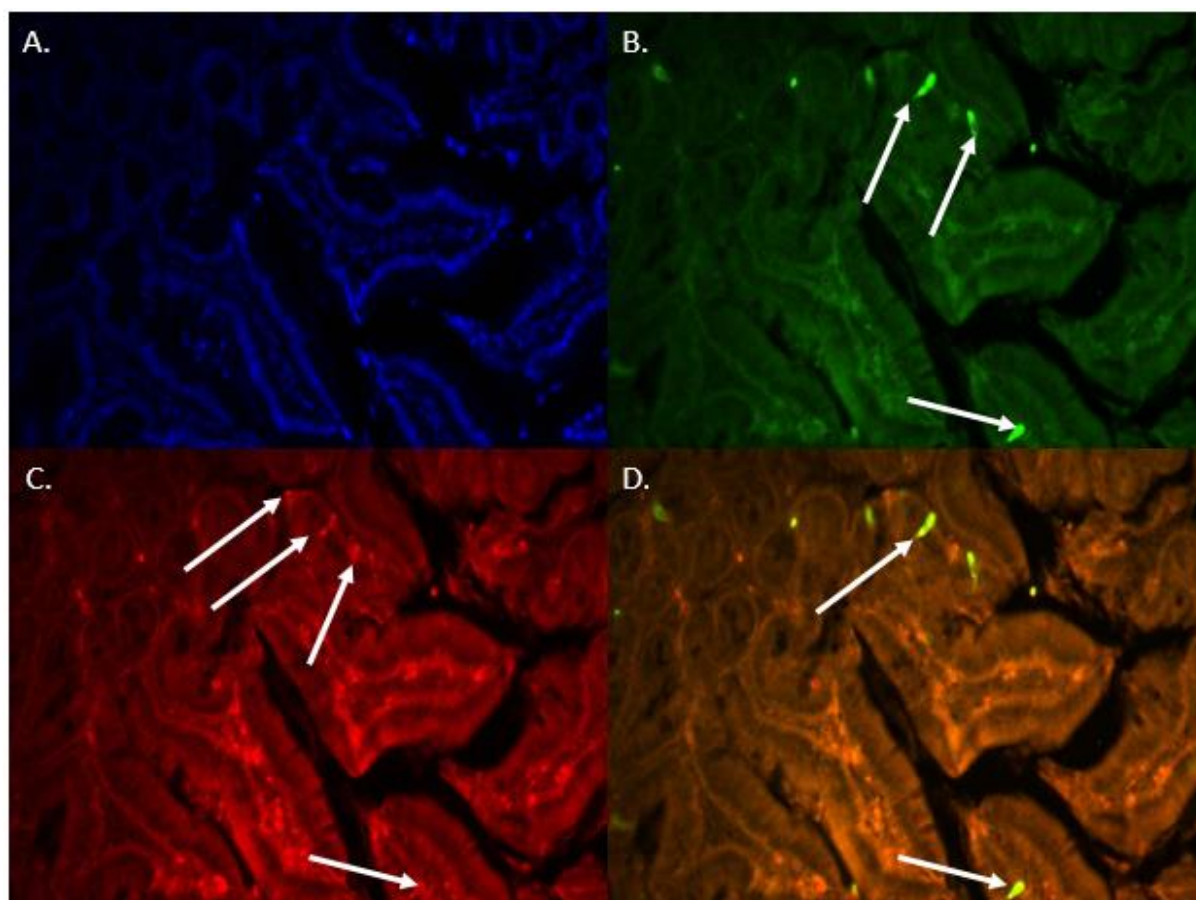
Distribution of (A) DAPI, (B) PYY, (C) GPR55 and (D) combined fluorescence of PYY and GPR55 in STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.26: Immunofluorescence staining of PYY and GPR55 in lean NIH Swiss mouse small intestine



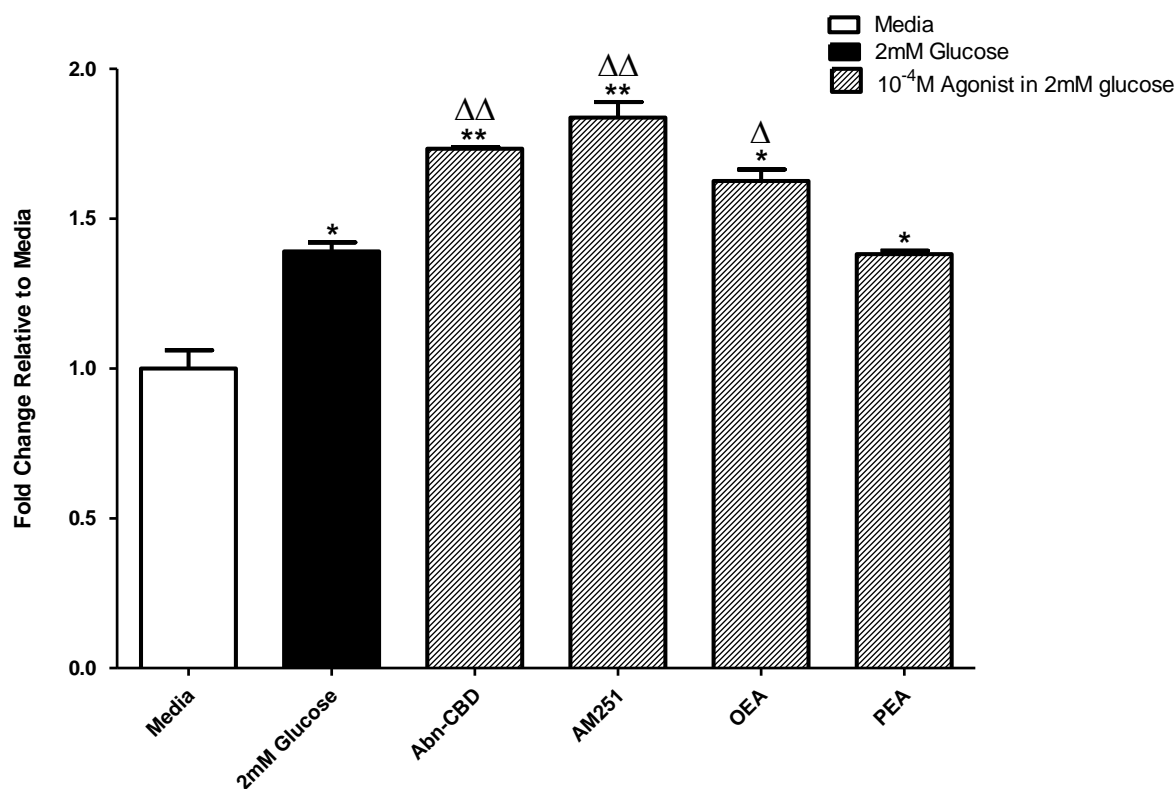
Distribution of (A) DAPI, (B) PYY, (C) GPR55 and (D) combined fluorescence of PYY and GPR55 in lean NIH Swiss Mouse Small Intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 6.27: Immunofluorescence staining of PYY and GPR55 in HFF NIH Swiss mouse small intestine



Distribution of (A) DAPI, (B) PYY, (C) GPR55 and (D) combined fluorescence of PYY and GPR55 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

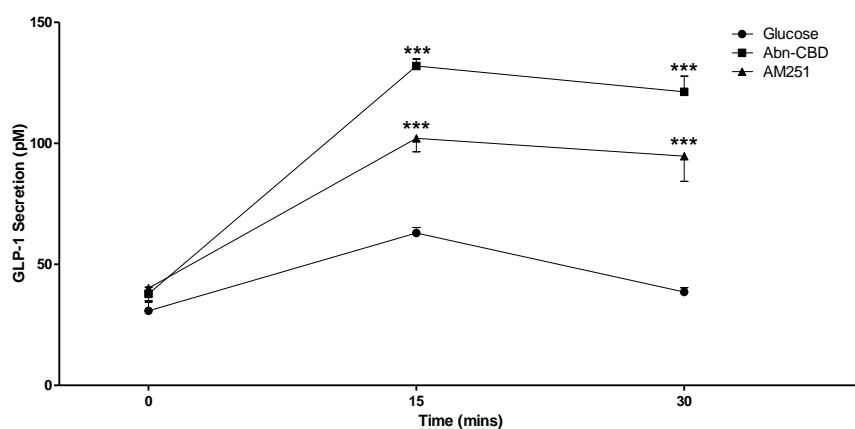
Figure 6.28: Effect of GPR55 Agonists on GPR55 gene expression in GLUTag cells



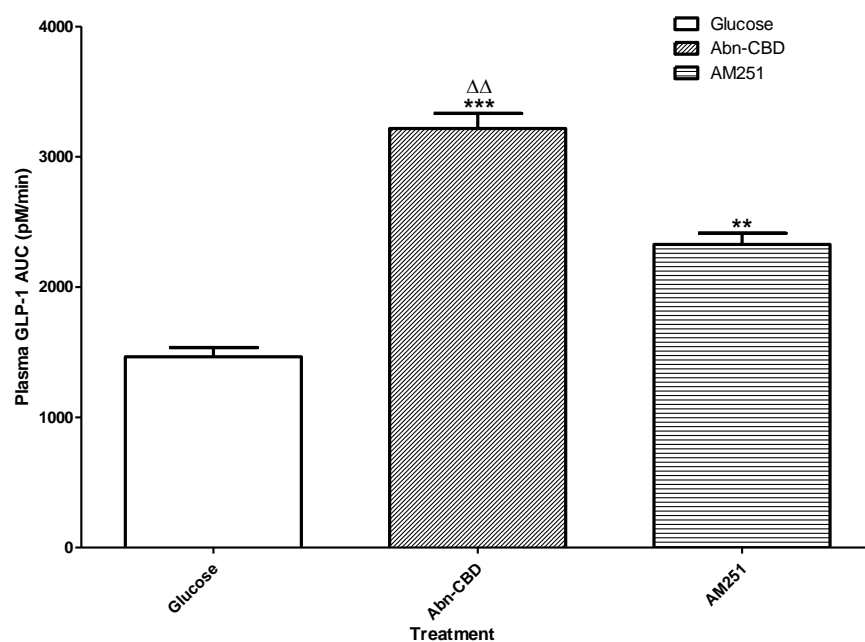
Effects of GPR55 agonists on the expression of the GPR55 gene in intestinal GLUTag cells at 2mM glucose. * $p < 0.05$ and ** $p < 0.01$ compared to untreated media control. Δ $p < 0.05$ and $\Delta\Delta$ $p < 0.01$ compared to vehicle glucose control (n=3).

Figure 6.29: Effects of GPR55 agonists on plasma GLP-1 in male Swiss TO mice

A.



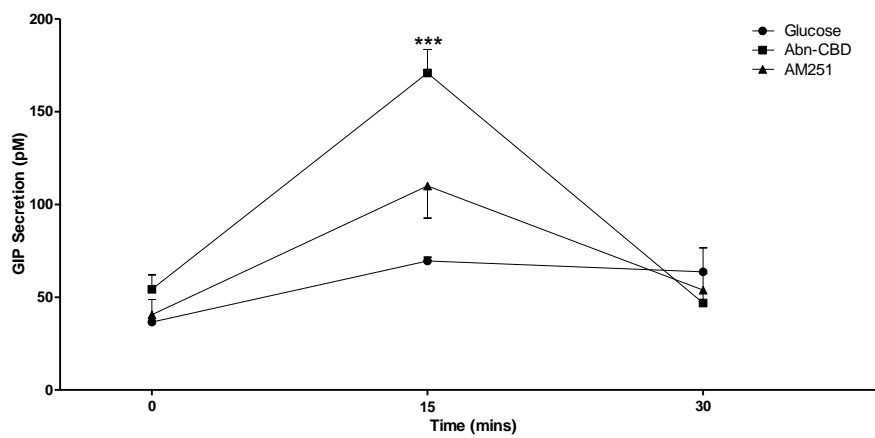
B.



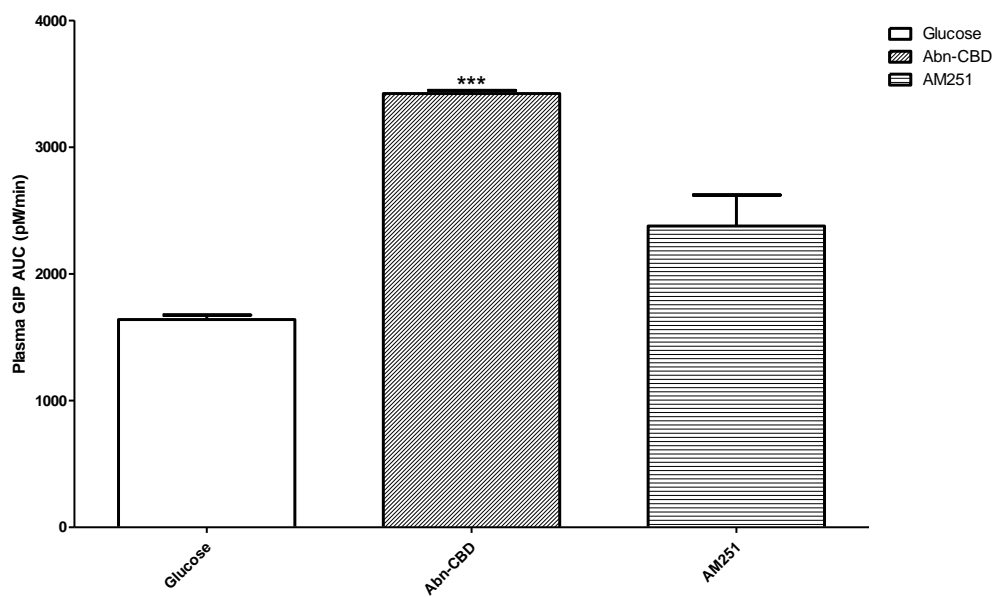
Glucose (18mmol/kg BW) or glucose in combination with a GPR55 agonist (0.1μmol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GLP-1 of mice treated with GPR55 agonists (B) AUC of A. **p<0.01 and ***p<0.001 compared to glucose treatment alone. ΔΔ p<0.01 compared to AM251.

Figure 6.30: Effects of GPR55 agonists on plasma GIP in male Swiss TO mice

A.



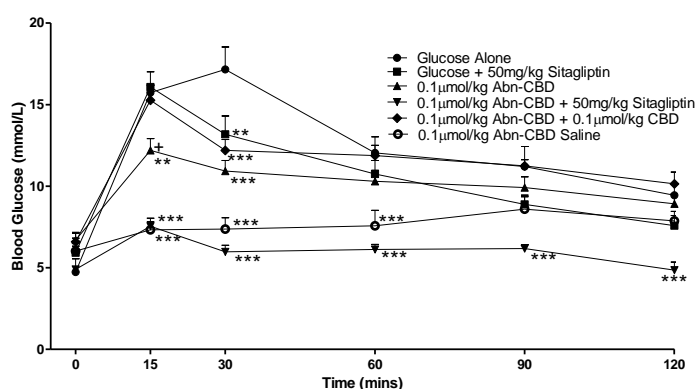
B.



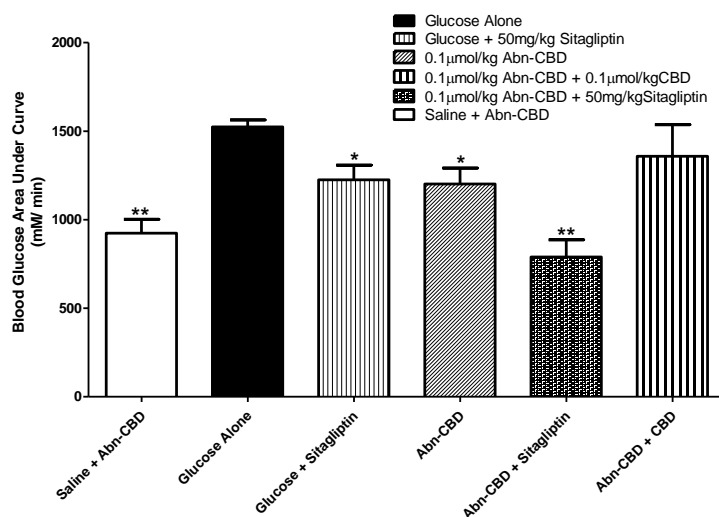
Glucose (18mmol/kg BW) or glucose in combination with a GPR55 agonist (0.1 μ mol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GIP of mice treated with GPR55 agonists (B) AUC of A. ***p<0.001 compared to glucose treatment alone.

Figure 6.31: Effects of GPR55 agonist Abn-CBD on blood glucose in male Swiss TO mice

A.



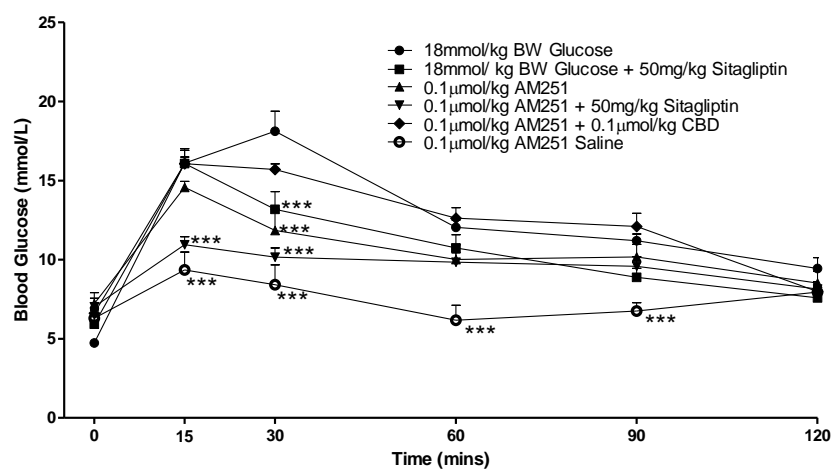
B.



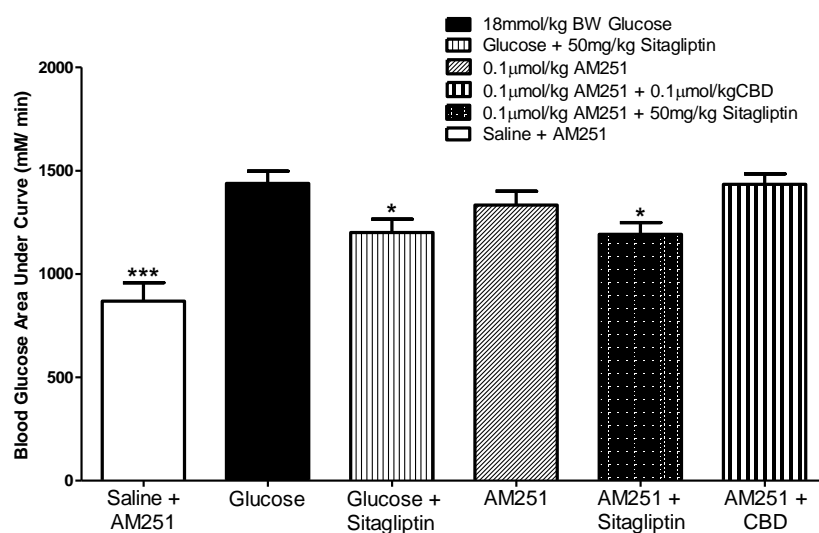
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with Abn-CBD (0.1µmol/kg BW), glucose in combination with Abn-CBD and the GPR55 antagonist CBD (0.1µmol/kg BW), glucose in combination with Abn-CBD and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with Abn-CBD were all administered orally to male Swiss TO mice. (A) Blood glucose of mice treated with Abn-CBD (B) AUC of A.

Figure 6.32: Effects of GPR55 agonist AM251 on blood glucose in male Swiss TO mice

A.



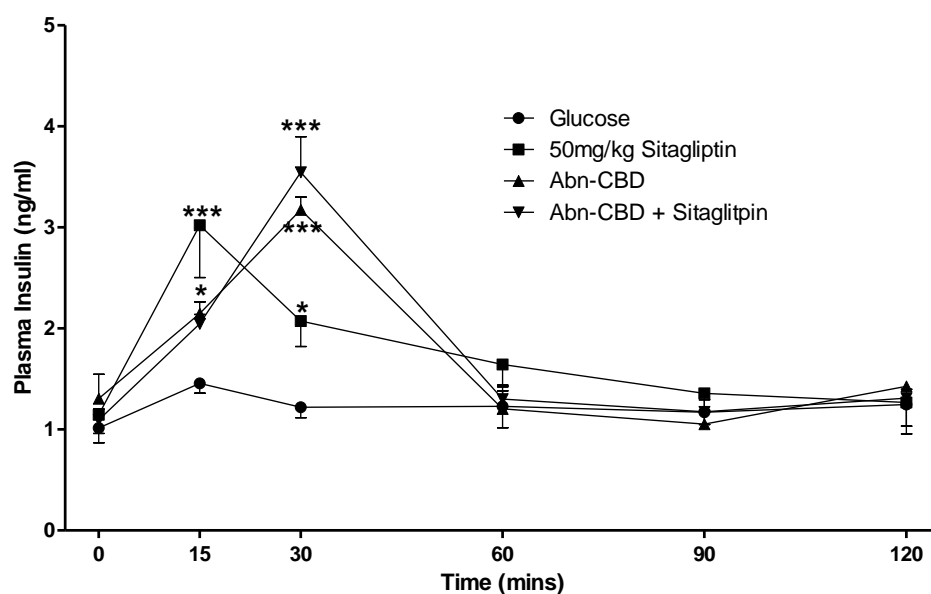
B.



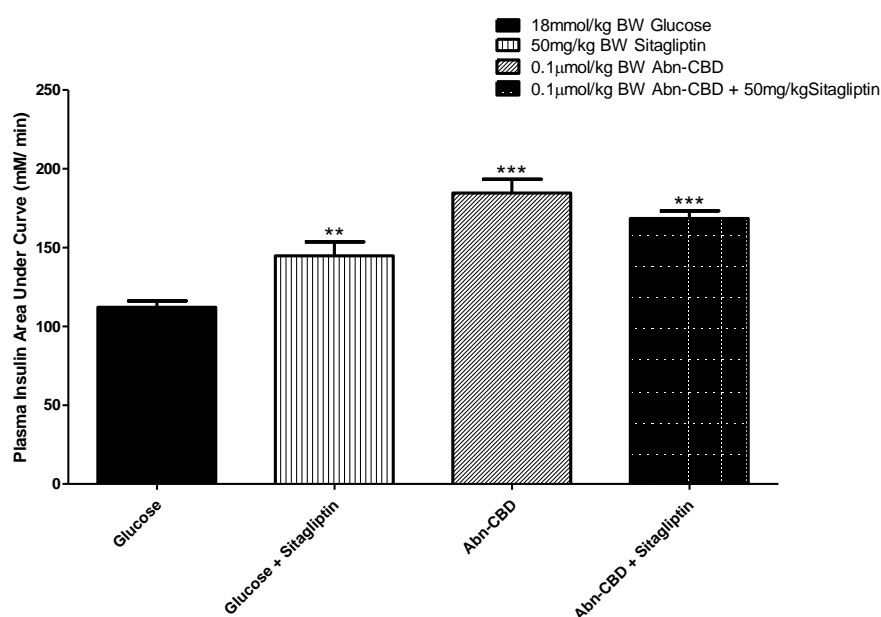
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with AM251 (0.1µmol/kg BW), glucose in combination with AM251 and the GPR55 antagonist CBD (0.1µmol/kg BW), glucose in combination with AM251 and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with a AM251 were all administered orally to male Swiss TO mice. (A) Blood glucose of mice treated with AM251 (B) AUC of A.

Figure 6.33: Effects of GPR55 agonists Abn-CBD on plasma insulin in male Swiss TO mice

A.



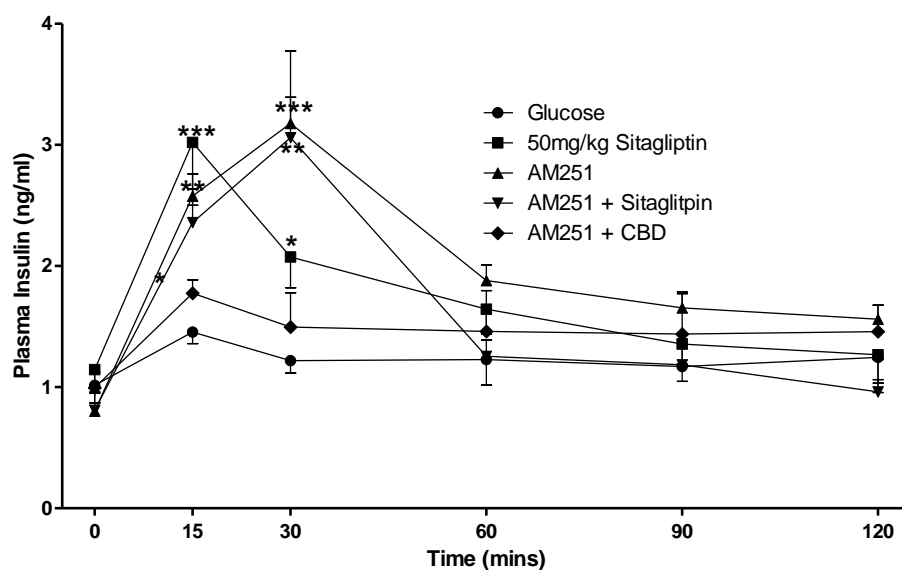
B.



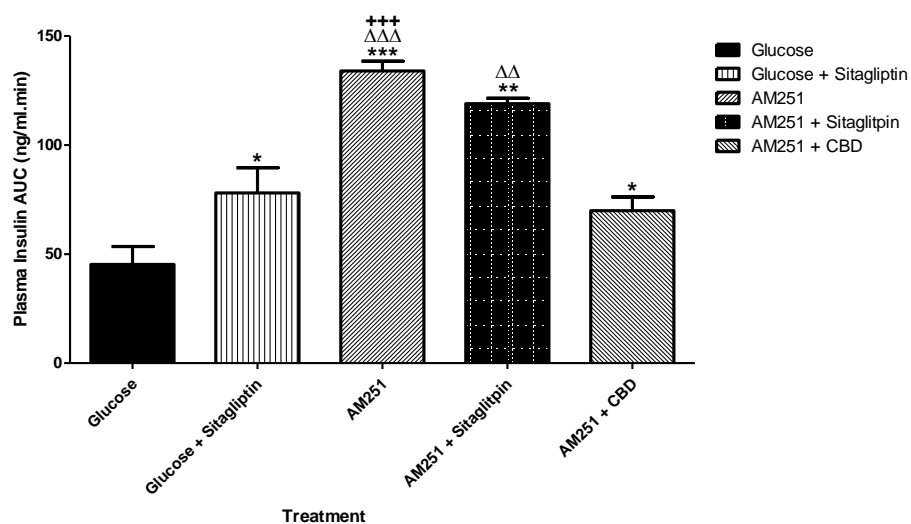
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with Abn-CBD (0.1 μ mol/kg BW), and glucose in combination with Abn-CBD and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with Abn-CBD (B) AUC of A. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to glucose.

Figure 6.34: Effects of GPR55 agonists Abn-CBD on plasma insulin in male Swiss TO mice

A.



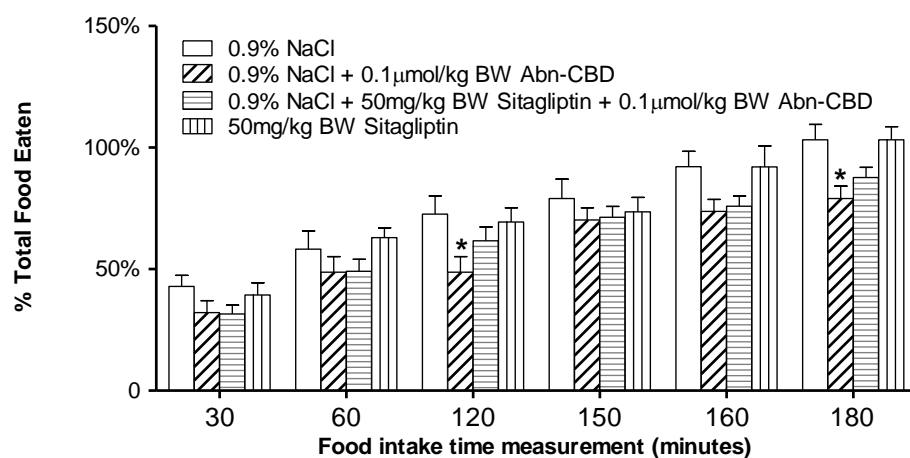
B.



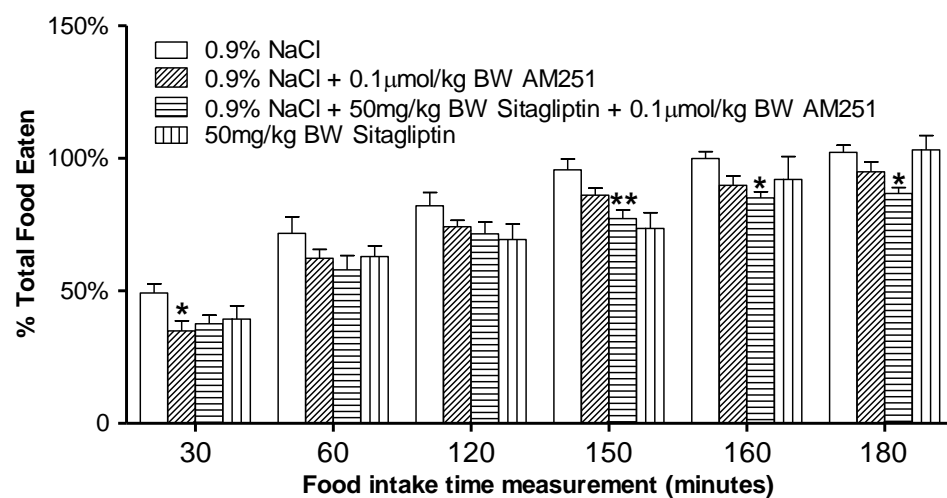
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with AM251 (0.1 μ mol/kg BW), and glucose in combination with AM251 and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with AM251 (B) AUC of A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose. +++ $p < 0.001$ compared to agonist in combination with CBD. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to glucose and Sitagliptin.

Figure 6.35: Effects of GPR55 agonists on acute feeding in trained male swiss TO mice

A.



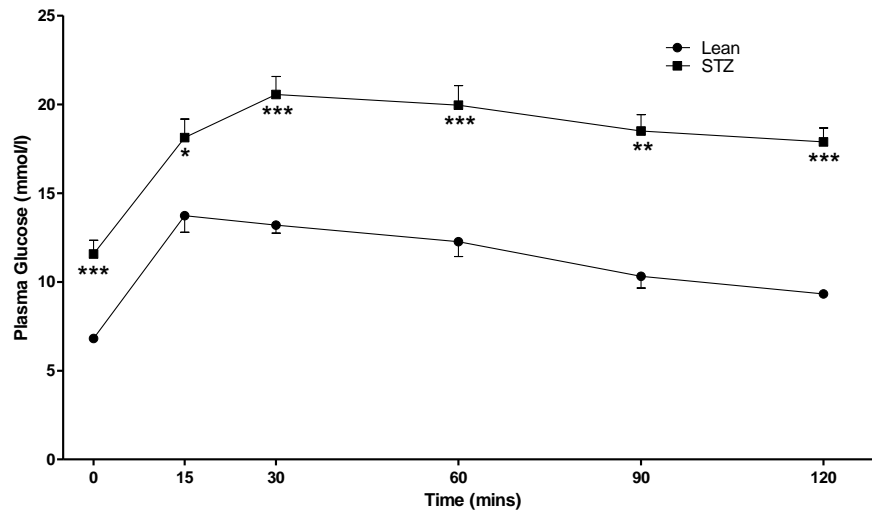
B.



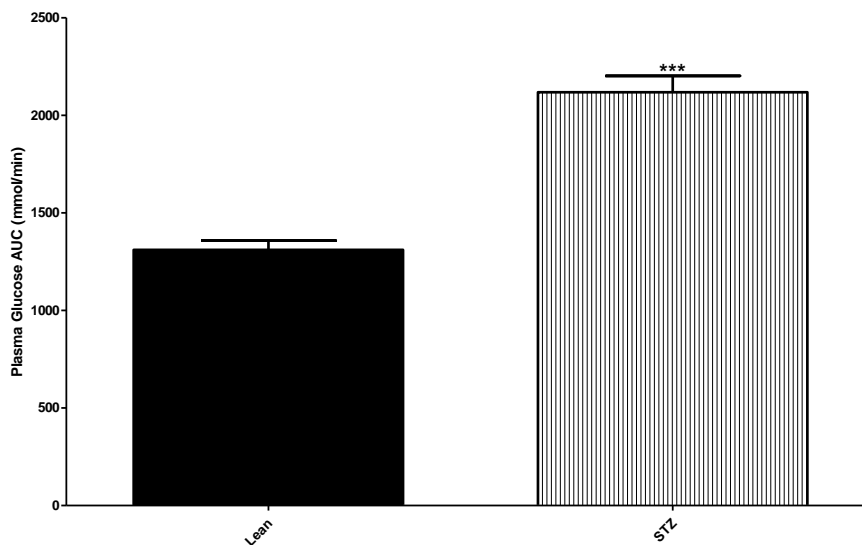
Saline (0.9%), saline and Abn-CBD (A) or AM251 (B) (0.1 $\mu\text{mol/kg}$ BW), saline with a GPR55 agonist and Sitagliptin (50mg/kg BW) or saline and Sitagliptin were administered orally to Swiss TO mice which had been trained to eat for 3 hrs daily (n=8). * $p < 0.05$ and ** $p < 0.01$ compared to saline.

Figure 6.36: Effect of multiple low dose streptozotocin on blood glucose in male Swiss TO mice

A.



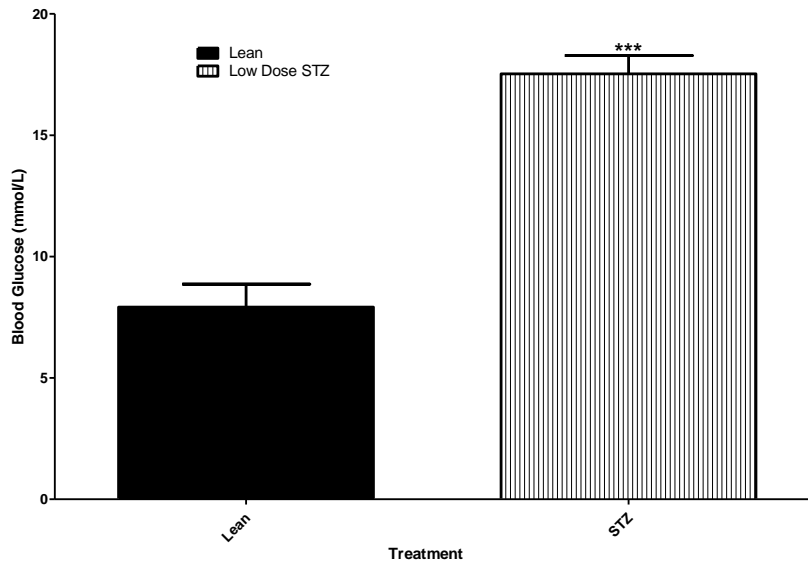
B.



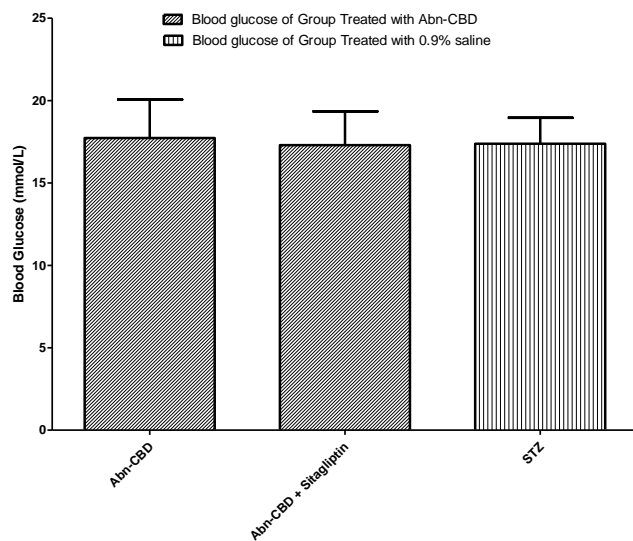
In order to induce diabetes, 4 hr fasted male Swiss TO mice were administered streptozotocin (40mg/kg BW, 0.1M sodium citrate, pH 4.5) via intraperitoneal injection. Reduced glucose tolerance was confirmed 14 days after the initial injection of streptozotocin (day 1) via oral glucose tolerance test. Glucose (18mmol/kg body weight) was administered orally to either lean or streptozotocin treated Swiss TO mice (n=6 for lean mice, n=12 for streptozotocin treated mice). (A) Blood glucose (B) AUC of A

Figure 6.37: Effect of multiple low dose streptozotocin on non-fasting blood glucose in male Swiss TO mice

A.

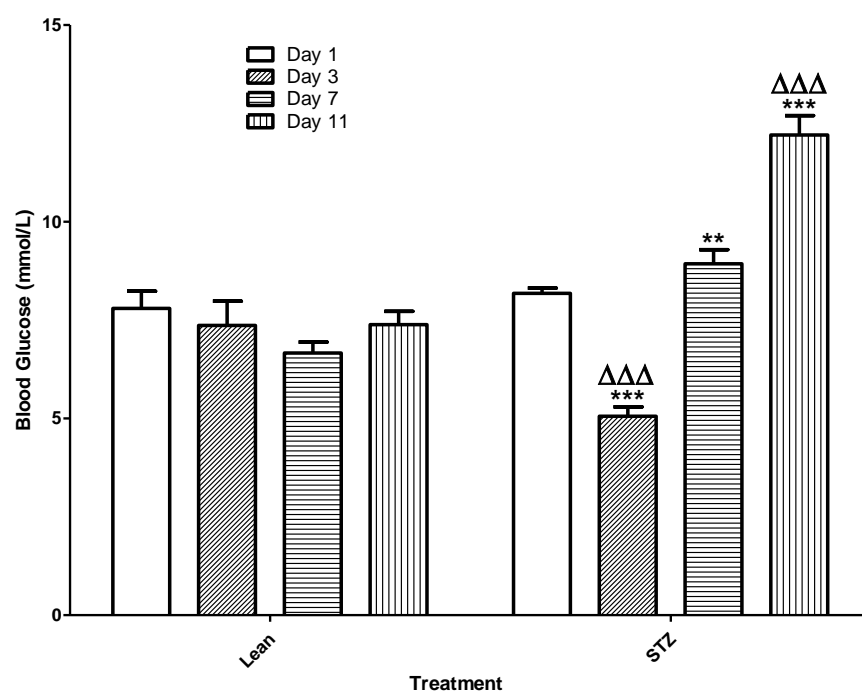


B.



In order to induce diabetes, 4 hr fasted male Swiss TO mice were administered streptozotocin (40mg/kg BW, 0.1M sodium citrate, pH 4.5) via intraperitoneal injection. Non-fasting blood glucose was measured 14 days after initial injection (n=6 for lean and n=12 for streptozotocin induced diabetic mice) (A) Non fasting blood glucose of mice 14 days after initial treatment (B) Non-fasting blood glucose levels between treatment groups of streptozotocin treated mice when separated 14 days after initial treatment. Mice were separated to ensure no significant differences between groups before treatment commenced.

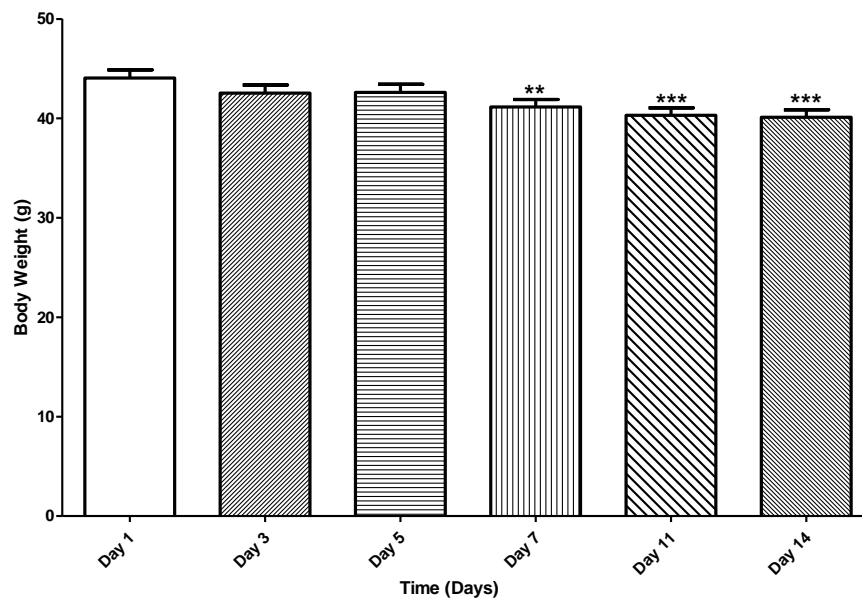
Figure 6.38: Effects of multiple low dose streptozotocin on blood glucose in 4 hr fasted male Swiss TO mice



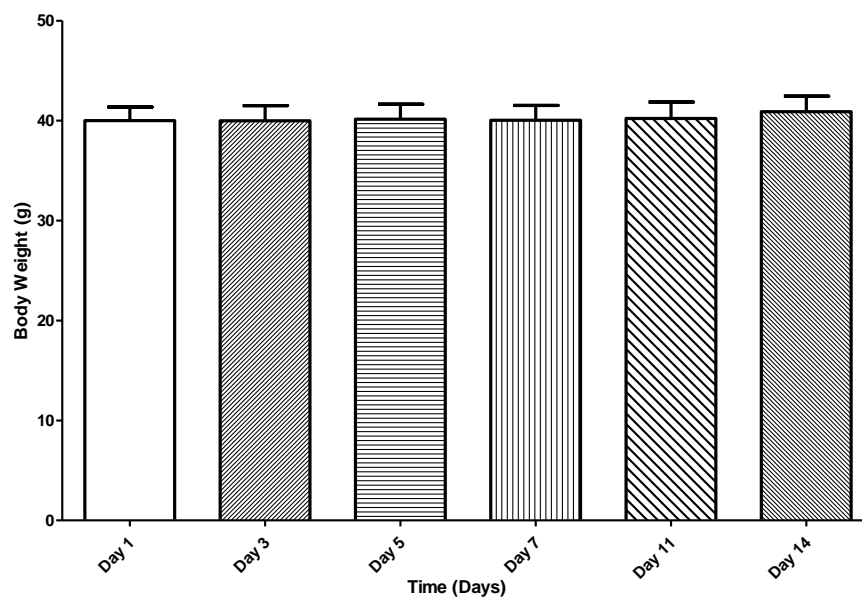
Male Swiss TO mice were fasted for 4 hrs prior to streptozotocin treatment to induce diabetes (n=32) or 0.9% saline (n=6) via intraperitoneal injection. Blood glucose was measured prior to injection.

Figure 6.39: Effects of multiple low dose streptozotocin on body weight in male Swiss TO mice

A.



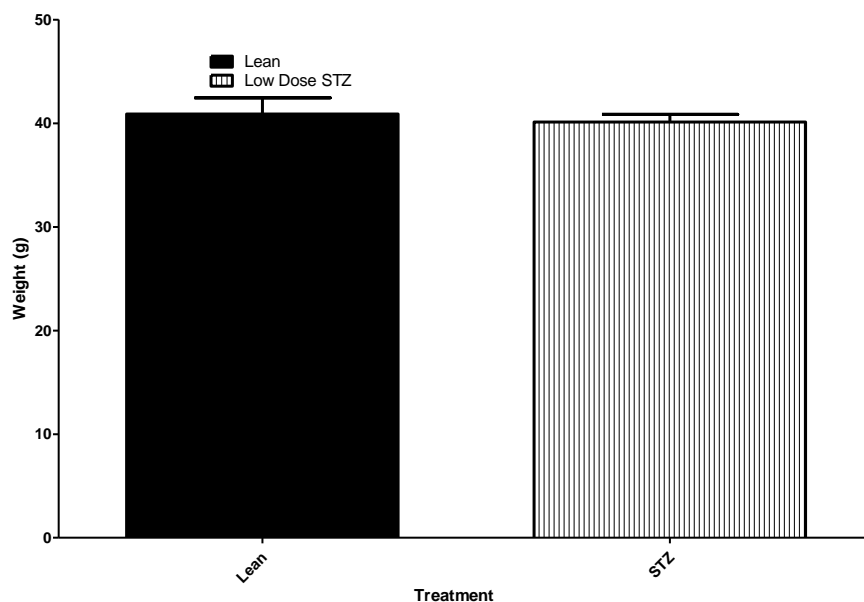
B.



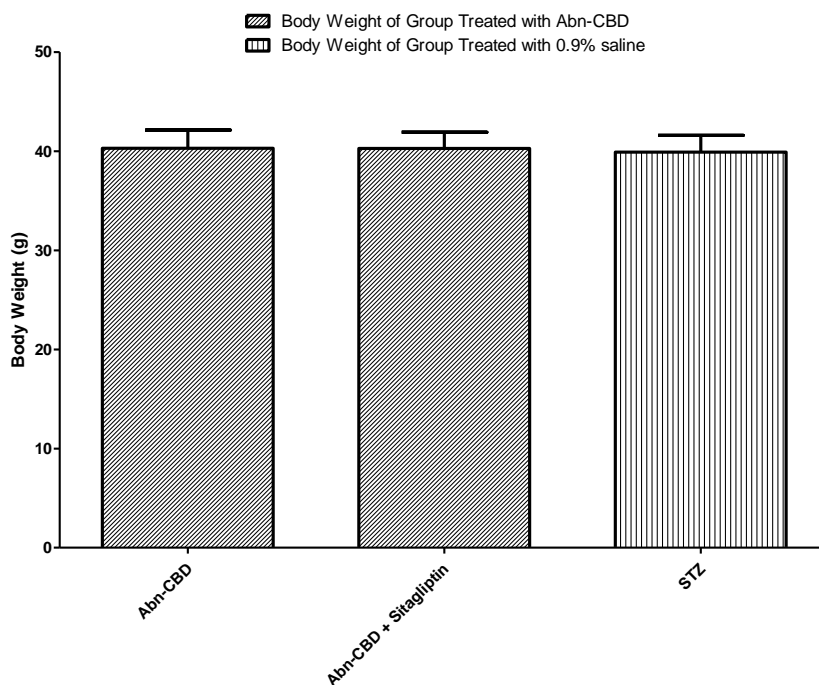
Body weights of both streptozotocin treated (A) and lean male Swiss TO mice (B) was monitored prior to injection (n=32 for streptozotocin treated mice, n=6 for lean mice). Lean mice received intraperitoneal injections of 0.9% saline in place of streptozotocin. ***p<0.001 compared to day 1.

Figure 6.40: Differences in body weight between treatment groups of streptozotocin induced diabetic male Swiss TO mice

A.



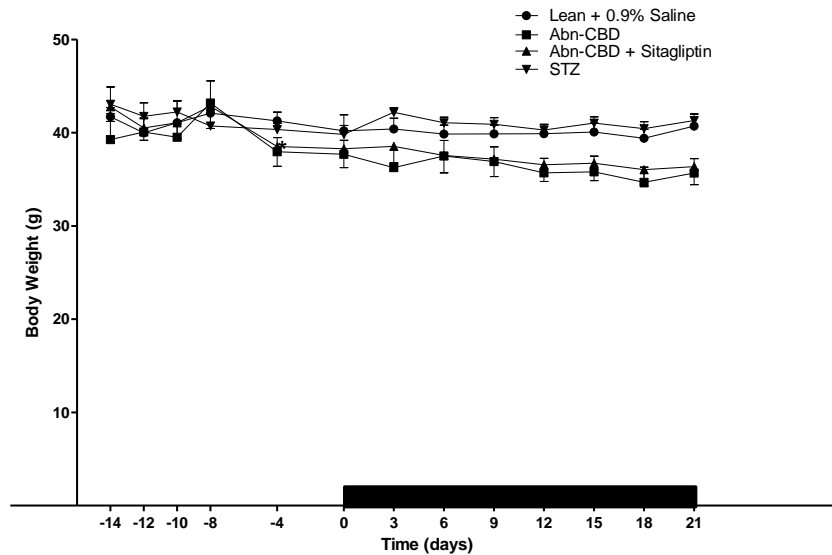
B.



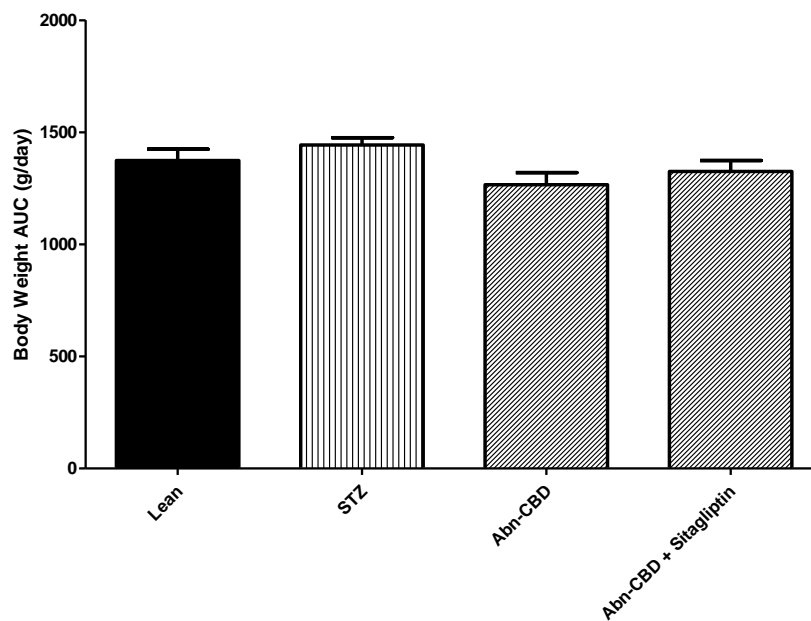
Body weight was compared between lean and streptozotocin treated mice 14 days after initial injection (A) and body weights were checked prior to initial agonist treatment (B).

Figure 6.41: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on body weight in multiple low dose streptozotocin mice

A.



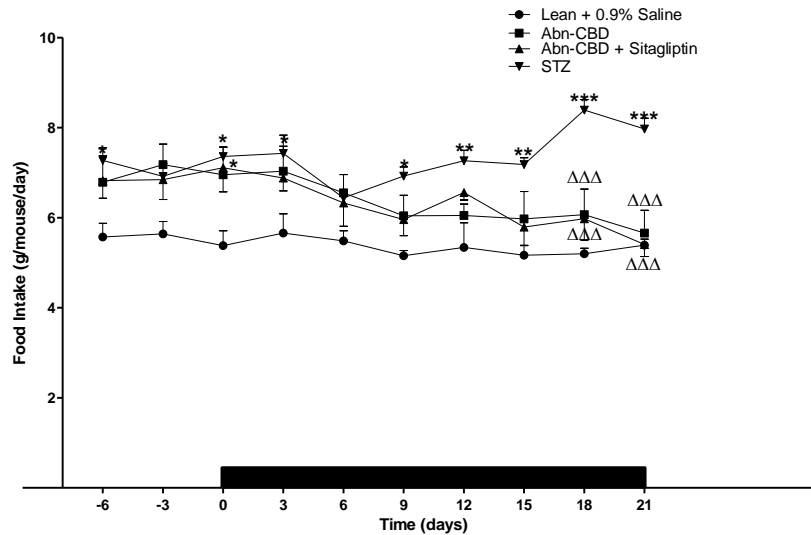
B.



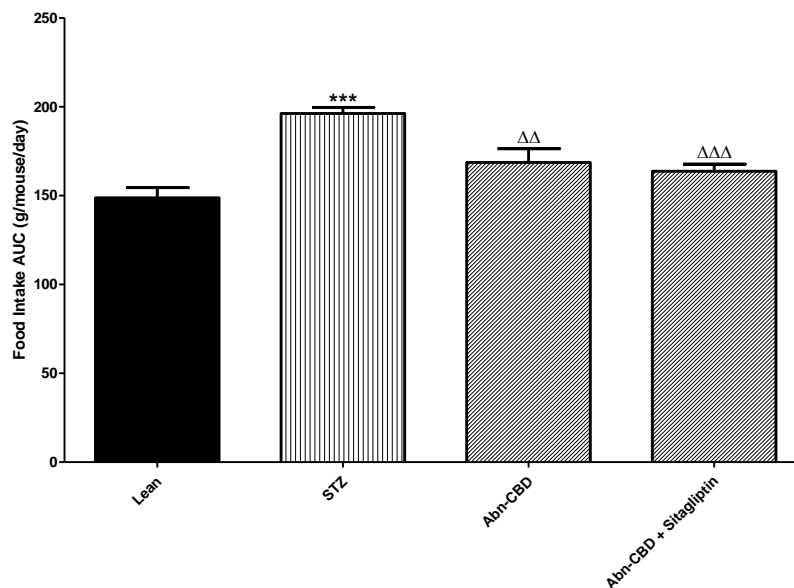
Effect of daily oral administration of saline (0.9%), Abn-CBD monotherapy (0.1 μ mol/kg body weight) and Abn-CBD (0.1 μ mol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) on body weight. (A) Body weight (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6).

Figure 6.42: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on food intake in multiple low dose streptozotocin mice

A.



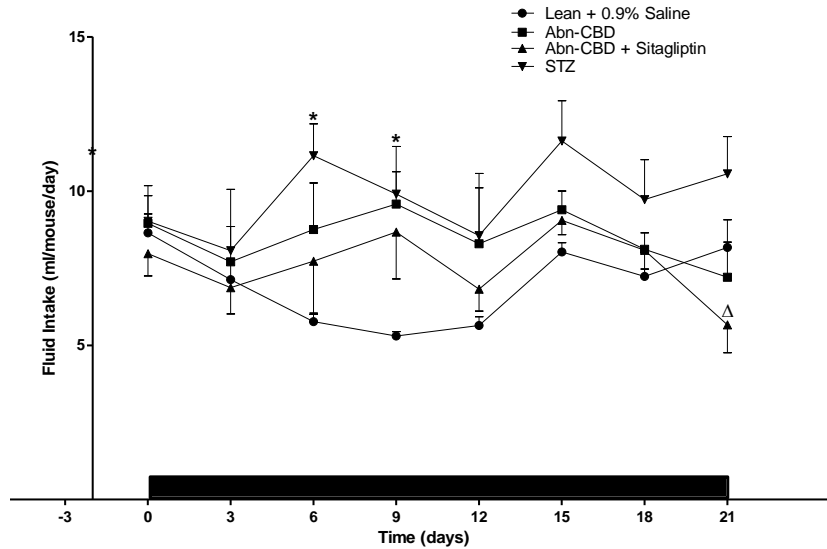
B.



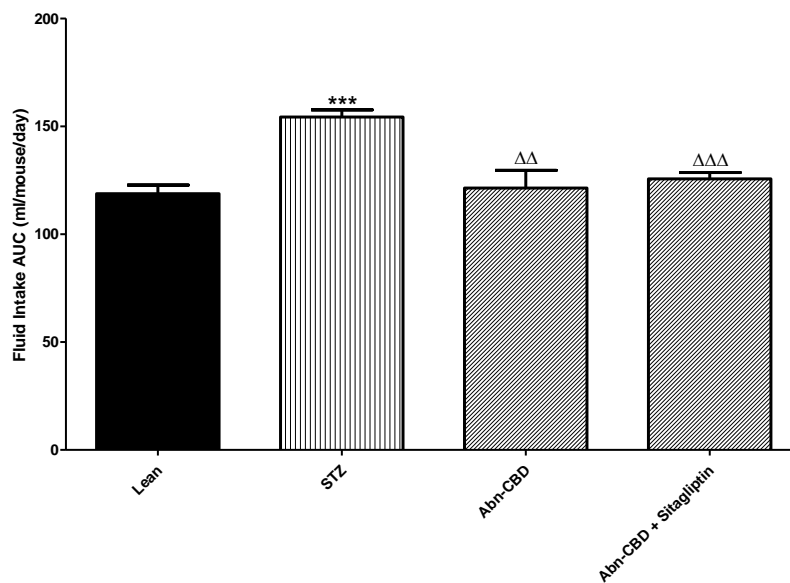
Effect of daily oral administration of saline (0.9%), Abn-CBD monotherapy (0.1 μ mol/kg body weight) and Abn-CBD (0.1 μ mol/kg body weight) combination therapy with Sitagliptin (50mg/kg bodyweight) on food intake. (A) Food intake (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin induced diabetic mice.

Figure 6.43: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on fluid intake in multiple low dose streptozotocin mice

A.



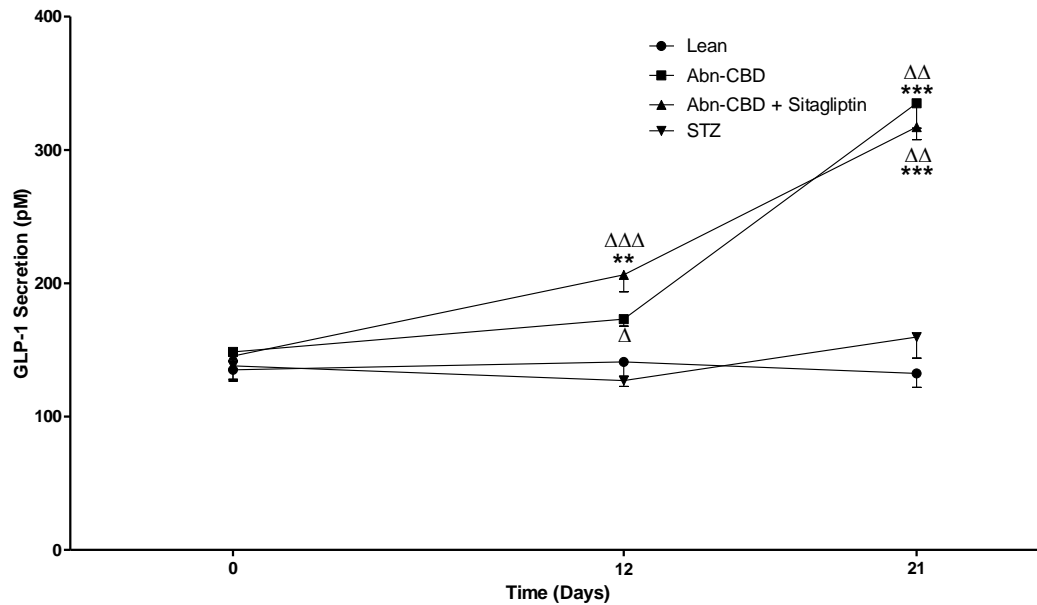
B.



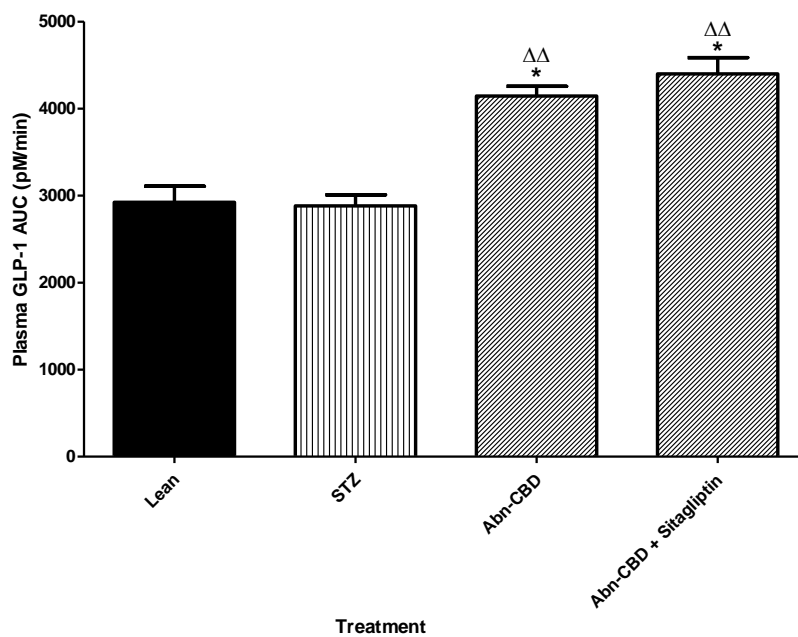
Effect of daily oral administration of saline (0.9%), Abn-CBD monotherapy (0.1μmol/kg body weight) and Abn-CBD (0.1μmol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) on fluid intake. (A) Fluid intake (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean ± SEM (n=6). * p<0.05 and *** p<0.001 compared to lean mice. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to the streptozotocin group.

Figure 6.44: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on plasma GLP-1 in multiple low dose streptozotocin mice

A.



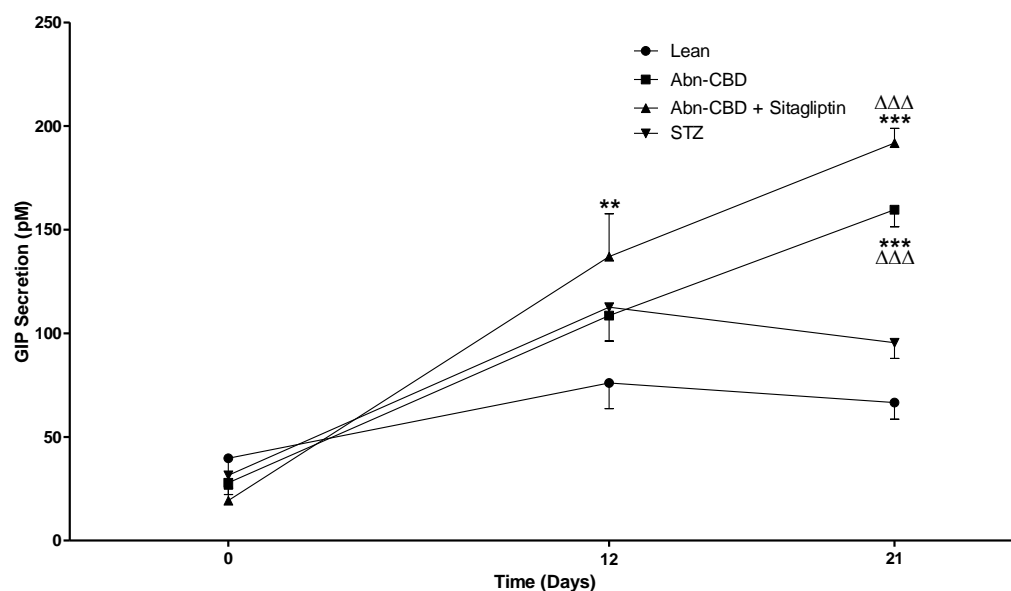
B.



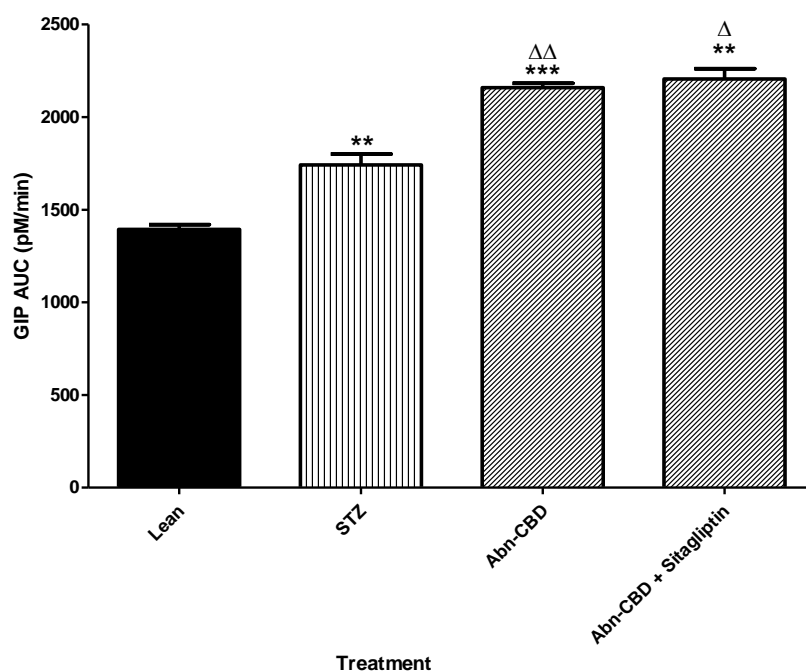
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) on (A) Plasma GLP-1 (B) AUC of A. ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin group.

Figure 6.45: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on plasma GIP in multiple low dose streptozotocin mice

A.



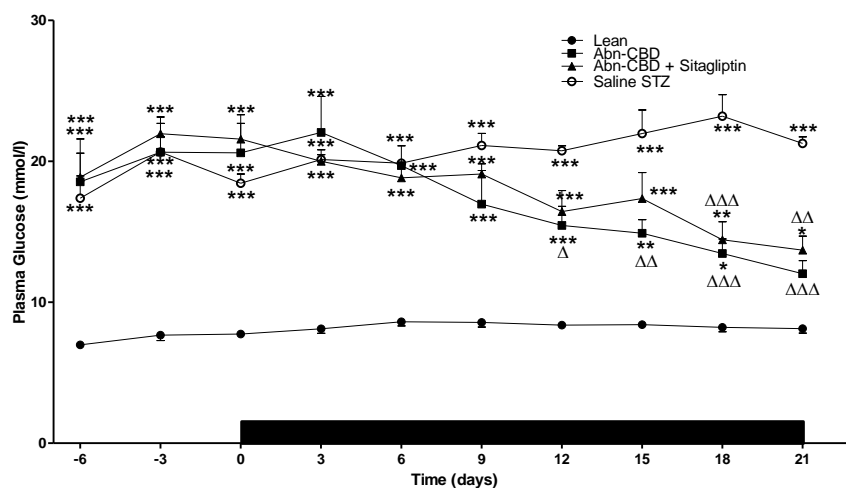
B.



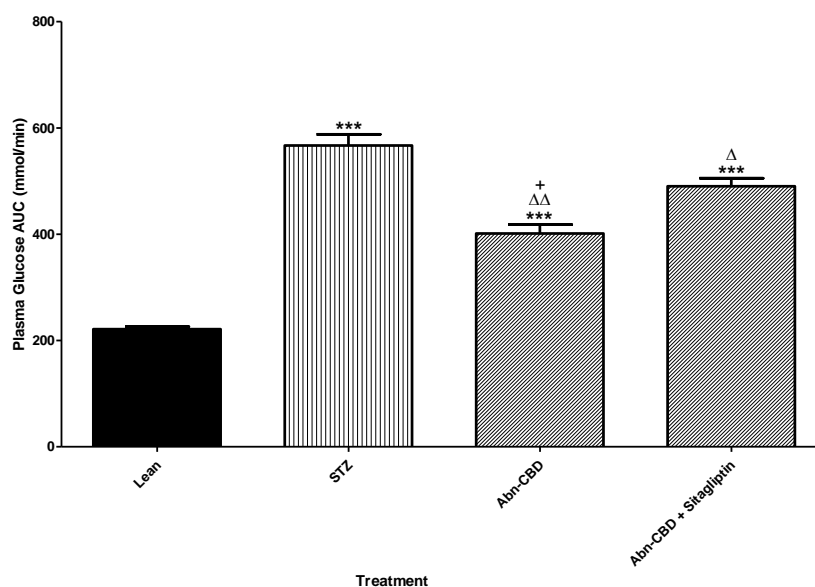
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) on (A) Plasma GIP of mice treated with GPR55 agonists (D) AUC of C. ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. Δ $p < 0.05$, $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin group.

Figure 6.46: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on blood glucose in multiple low dose streptozotocin mice

A.



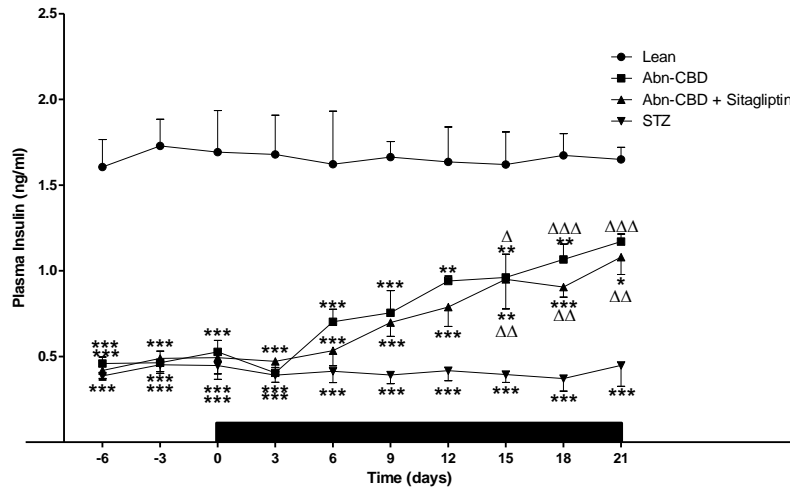
B.



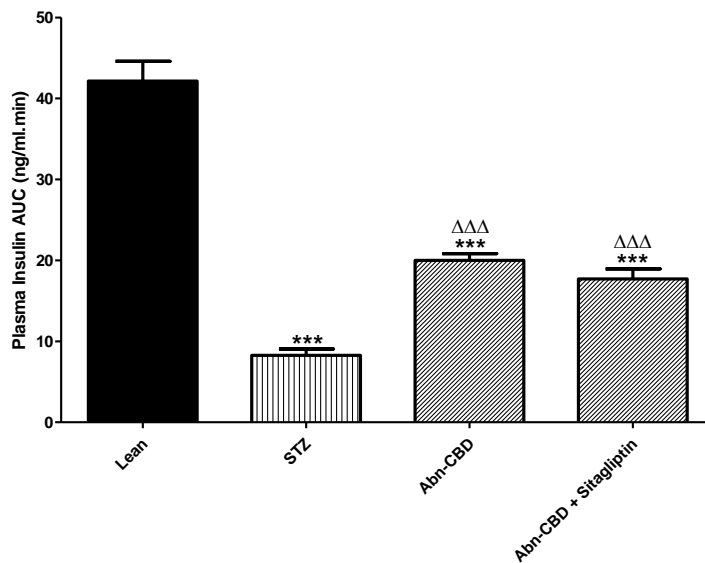
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg BW) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg BW) on non-fasting blood glucose. (A) Non-fasting blood glucose (B) AUC of A. Parameters obtained before and during the 21 day treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the lean group. Δ $p < 0.05$, $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to the streptozotocin group.

Figure 6.47: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on plasma insulin in multiple low dose streptozotocin mice

A.



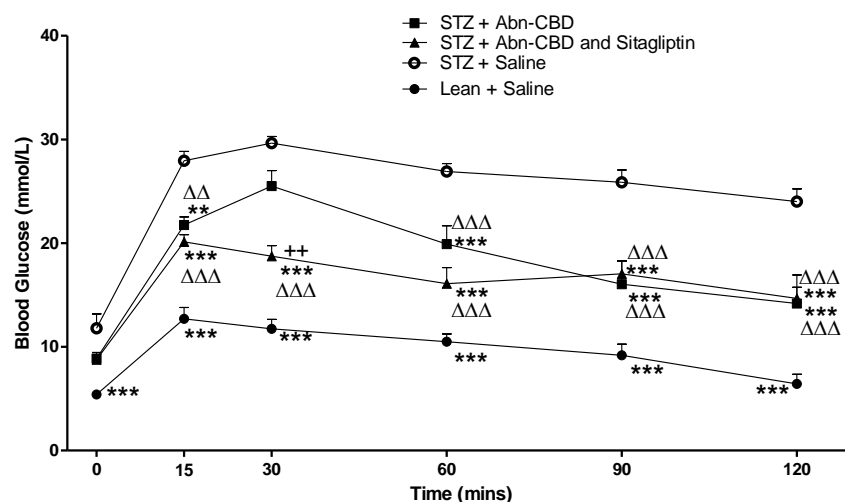
B.



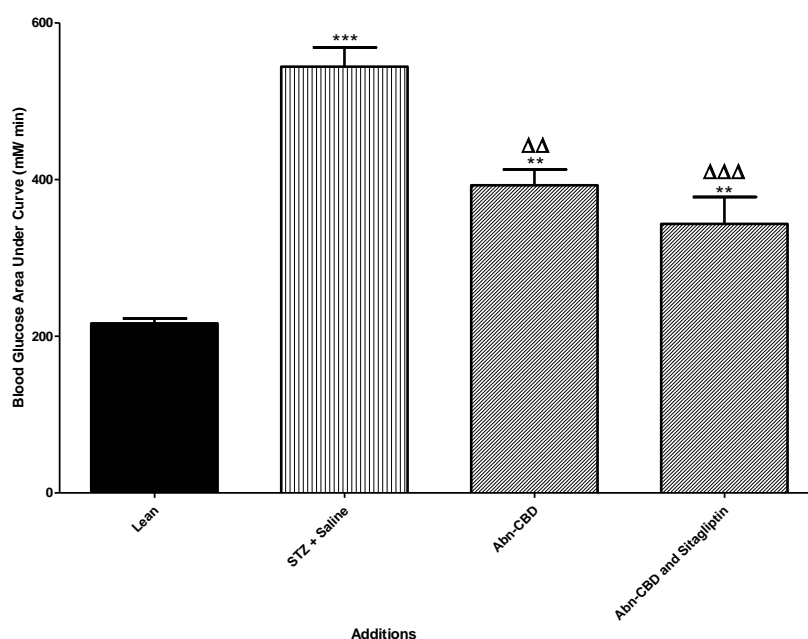
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg BW) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg BW) on non-fasting plasma insulin (A) Non-fasting plasma insulin. Parameters obtained before and during the 21 day treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the lean group. Δ $p < 0.05$, $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to the streptozotocin group.

Figure 6.48: Effects of long term oral treatment of GPR55 agonist Abn-CBD on glucose tolerance in multiple low dose streptozotocin mice

A.



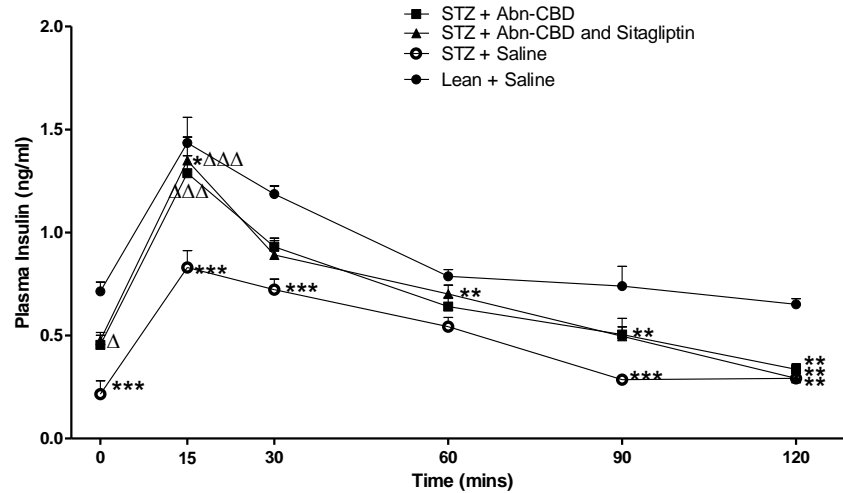
B.



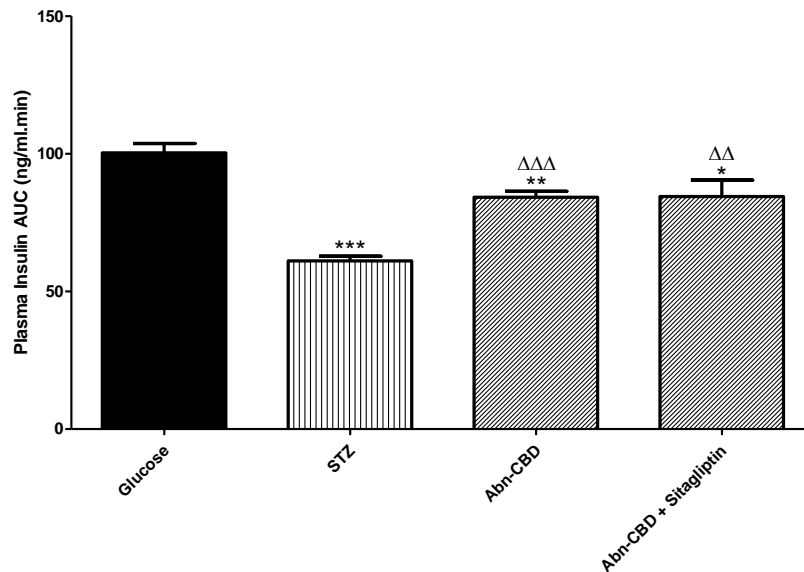
Oral glucose tolerance test (18mmol/kg body weight) was performed 21 days after treatment with saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg BW) to 18 hr fasted Swiss TO mice. (A) Blood glucose (B) AUC of A. Results are the mean \pm SEM (n=6). ** p<0.01 and *** p<0.001 compared to glucose alone. $\Delta\Delta$ p<0.01 and $\Delta\Delta\Delta$ p<0.001 compared to the streptozotocin group. ++ p<0.01 compared to agonist monotherapy.

Figure 6.49: Effects of long term oral treatment of GPR55 agonist Abn-CBD on acute insulin secretion in multiple low dose streptozotocin mice

A.



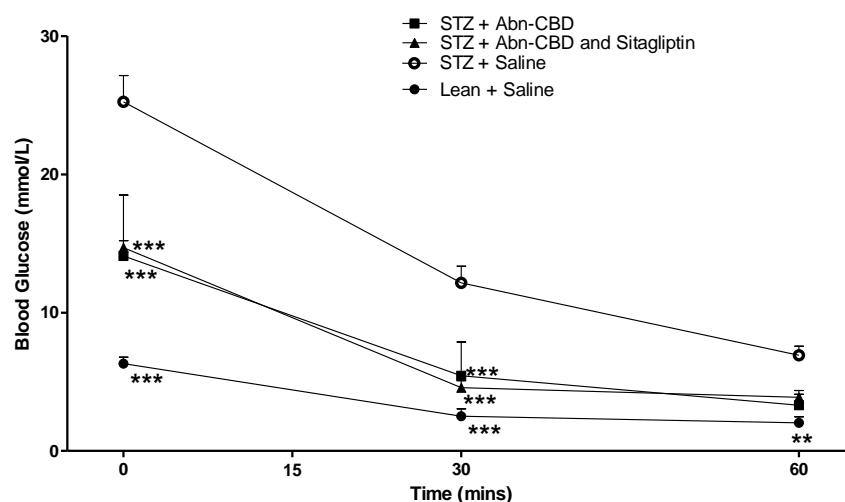
B.



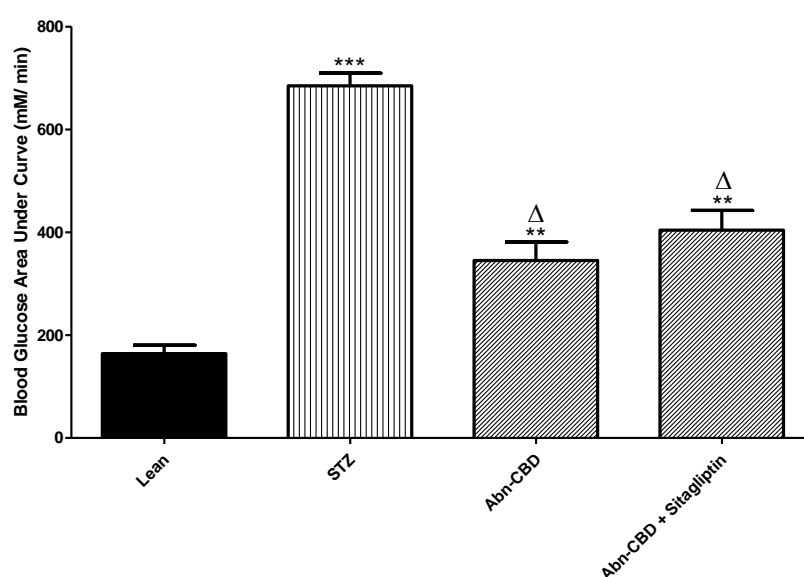
Oral glucose tolerance test (18mmol/kg body weight) was performed 21 days after treatment with saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg BW) to 18 hr fasted Swiss TO mice. (A) Plasma insulin (B) AUC of A. Results are the mean \pm SEM (n=6). ** p<0.01 and *** p<0.001 compared to lean mice. Δ p<0.05, $\Delta\Delta$ p<0.01 and $\Delta\Delta\Delta$ p<0.001 compared to the streptozotocin group.

Figure 6.50: Effects of long term daily oral treatment GPR55 agonist Abn-CBD on insulin sensitivity in multiple low dose streptozotocin mice

A.



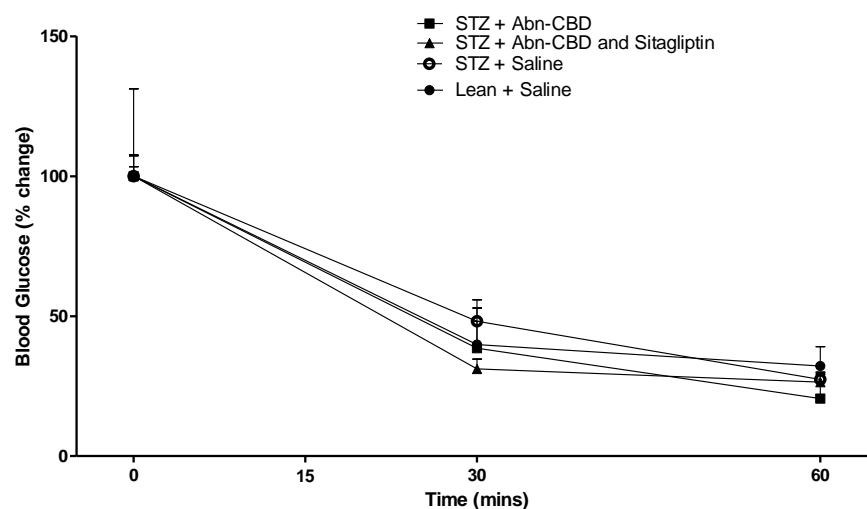
B.



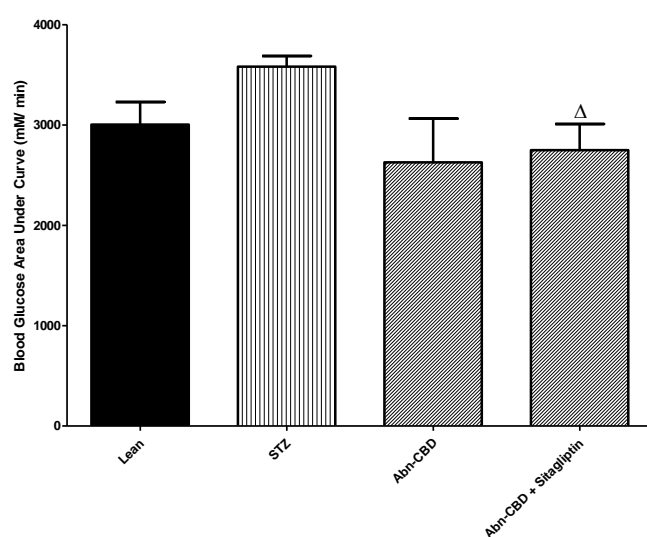
Insulin sensitivity (25 U/kg body weight, I. P. injection dissolved in 0.9% NaCl) was performed 21 days after treatment with saline, Abn-CBD monotherapy (0.1μmol/kg body weight) or Abn-CBD (0.1μmol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) in non-fasted Swiss TO mice (n=6). (A) Blood glucose, (B) AUC of blood glucose. Results are the mean ± SEM (n=6). ** p<0.01 and *** p<0.001 compared to lean. Δ p<0.05 compared to the streptozotocin group.

Figure 6.51: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on insulin sensitivity in multiple low dose streptozotocin mice

A.



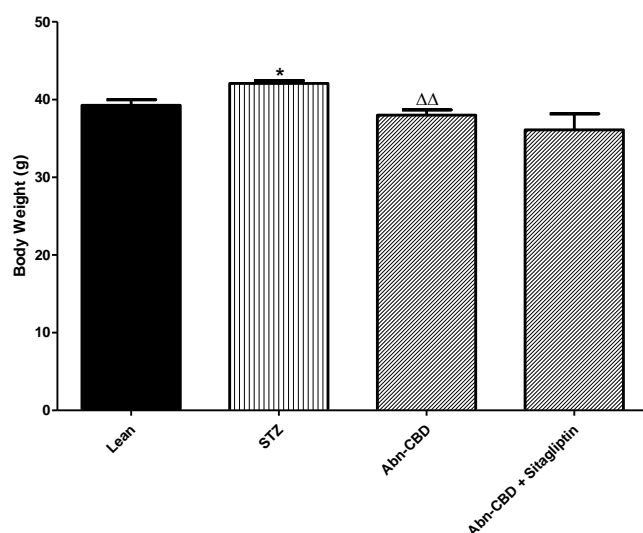
B.



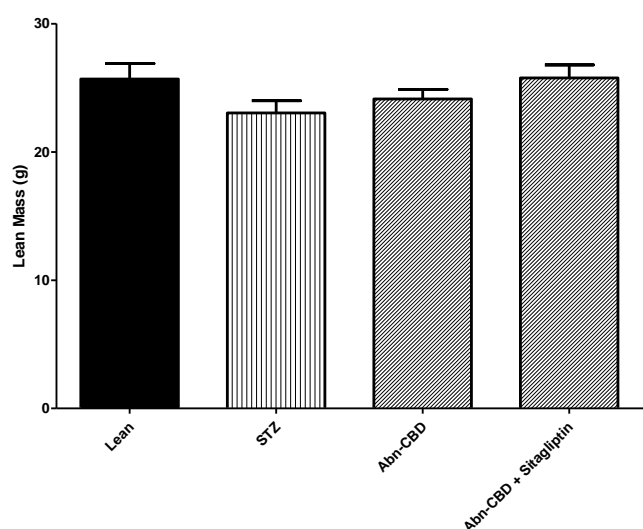
Insulin sensitivity (25 U/kg body weight, I. P. injection dissolved in 0.9% NaCl) was performed 21 days after treatment with saline, Abn-CBD monotherapy (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) combination therapy with Sitagliptin (50mg/kg body weight) in non-fasted Swiss TO mice (n=6). (A) Blood glucose (% change) (B) AUC of blood glucose (% change) are shown. Results are the mean \pm SEM (n=6). Δ $p < 0.05$ compared to the streptozotocin group.

Figure 6.52: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on body weight and lean mass as measured by DEXA scanning in multiple low dose streptozotocin mice

A.



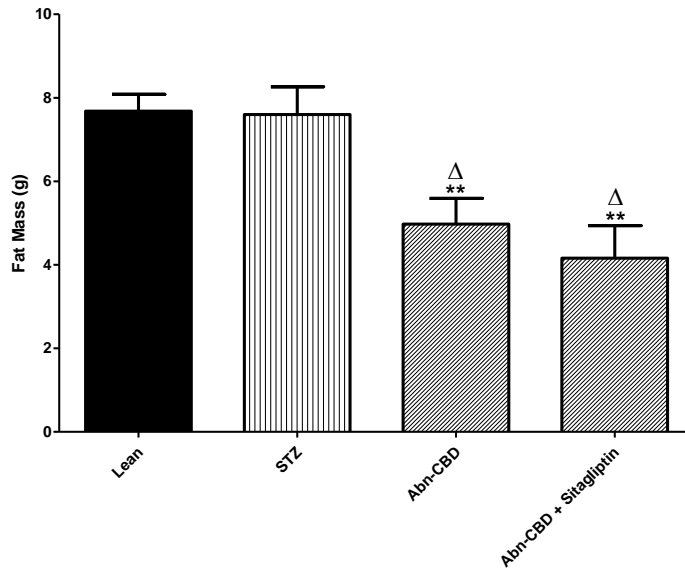
B.



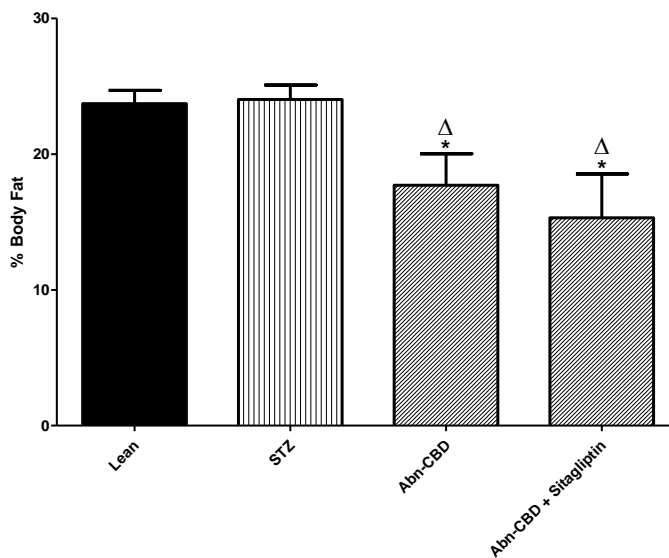
Effect of daily oral administration of saline, Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg body weight) on (A) body weight and (B) lean mass as measured by DEXA scanning following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean \pm SEM (n=6). * $p < 0.05$ compared to the lean group. $\Delta\Delta$ $p < 0.01$ compared to streptozotocin group.

Figure 6.53: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on fat mass and % body fat as measured by DEXA scanning in multiple low dose streptozotocin mice

A.



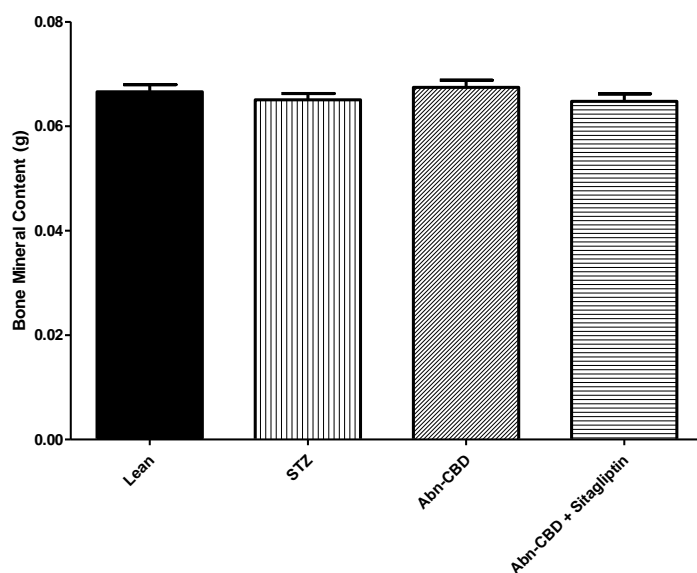
B.



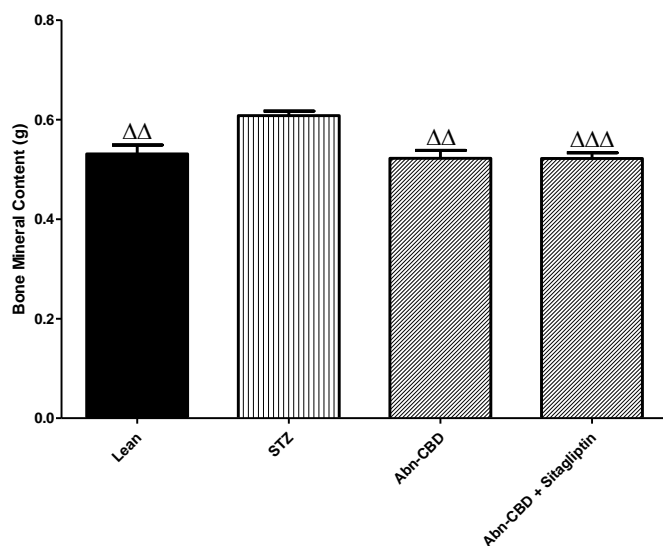
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg body weight) on (A) fat mass and (B) fat (% of bodyweight) as measured by DEXA scanning following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean \pm SEM (n=6). * p<0.05 and ** p<0.01 compared to the lean group. Δ p<0.05 compared to streptozotocin group.

Figure 6.54: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on bone mineral density and content as measured by DEXA scanning in multiple low dose streptozotocin mice

A.



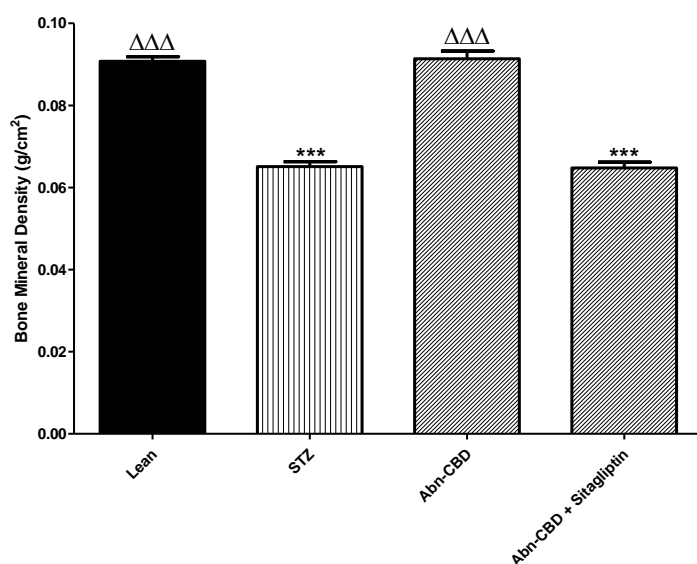
B.



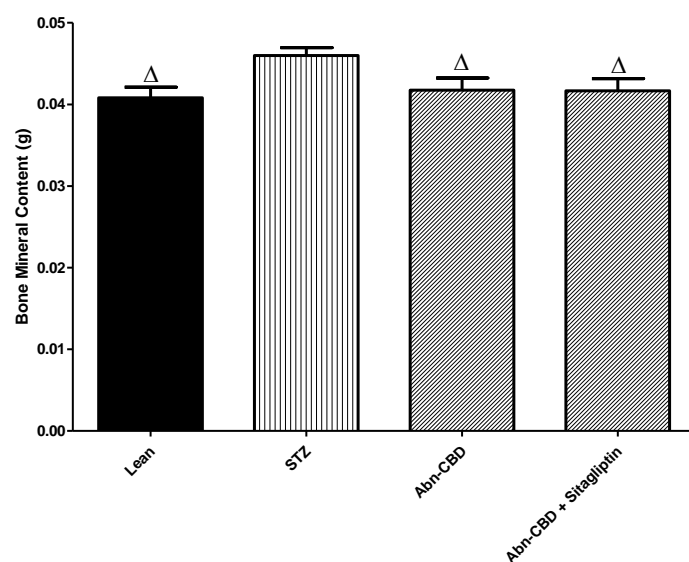
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1 μ mol/kg body weight) or Abn-CBD (0.1 μ mol/kg BW) in combination with Sitagliptin (50mg/kg body weight) on (A) bone mineral content and (B) bone mineral density as measured by DEXA scanning following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean \pm SEM (n=6). $\Delta\Delta$ p<0.01 and $\Delta\Delta\Delta$ p<0.001 compared to the streptozotocin group.

Figure 6.55: Effects of long term daily oral treatment of GPR55 agonist Abn-CBD on bone mineral density and content in the femur as measured by DEXA scanning in multiple low dose streptozotocin mice

A.



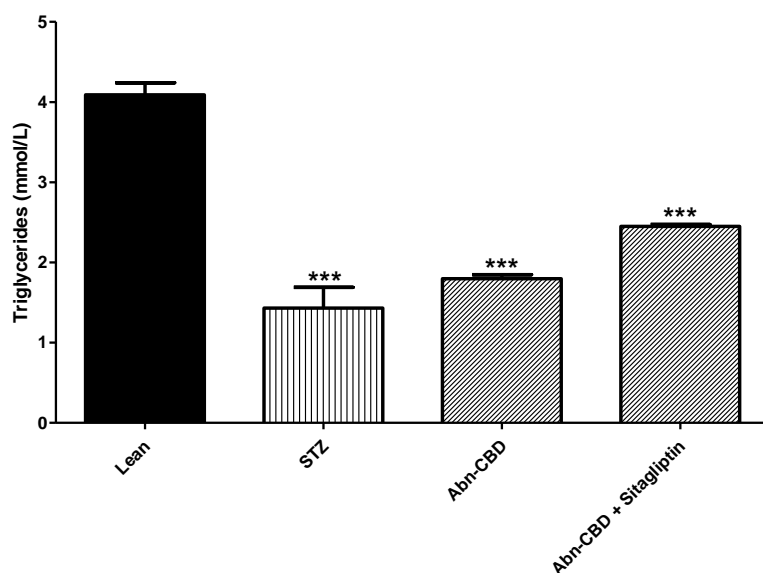
B.



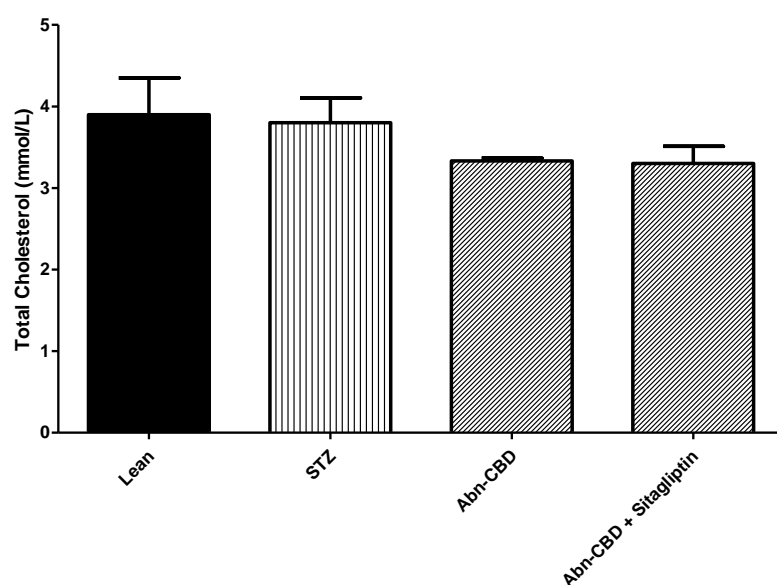
Effect of daily oral administration of saline (0.9%), Abn-CBD (0.1μmol/kg body weight) or Abn-CBD (0.1μmol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) on (A) bone mineral density and (B) bone mineral content in the femur as measured by DEXA scanning following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean ± SEM (n=6). ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to the streptozotocin group.

Figure 6.56: Effects of long term oral treatment of GPR55 agonist Abn-CBD on plasma triglycerides and total cholesterol in multiple low dose streptozotocin mice

A.



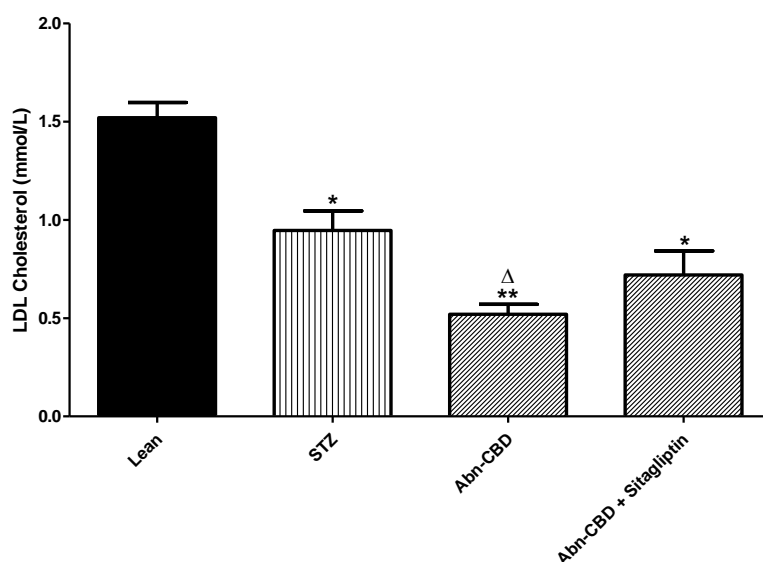
B.



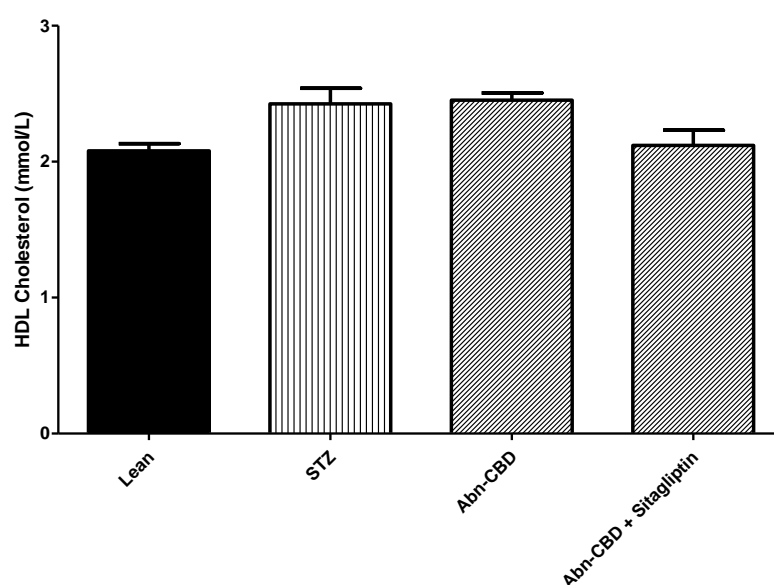
Effect of daily oral administration of saline (0.9%), Abn-CBD monotherapy (0.1 μ mol/kg body weight) and Abn-CBD (0.1 μ mol/kg BW) combination therapy with Sitagliptin (50mg/kg body weight) on (A) triglycerides and (B) total cholesterol following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean \pm SEM (n=6). *** p<0.001 compared to the lean group.

Figure 6.57: Effects of long term oral treatment of GPR55 agonist Abn-CBD on LDL and HDL cholesterol in multiple low dose streptozotocin mice

A.



B.



Effect of daily oral administration of saline (0.9%), Abn-CBD monotherapy (0.1 μ mol/kg body weight) and Abn-CBD (0.1 μ mol/kg BW) combination therapy with Sitagliptin (50mg/kg body weight) on (A) HDL cholesterol and (B) LDL cholesterol following 21 day treatment period in streptozotocin-induced diabetic mice. LDL was calculated using the Friedewald equation (LDL cholesterol = Total cholesterol – HDL cholesterol – (Triglycerides/5)). Values are mean \pm SEM (n=6). * p<0.05 and ** p<0.01 compared to lean mice. Δ p<0.05 compared to streptozotocin group.

Chapter 7

Determination of the effects of GPR119
activation on gastrointestinal hormone
secretion both *in vitro* and *in vivo*

7.1: Overview of results

GPR119 was identified through bioinformatical analysis and found to be present in various genomes including those of fish. The discovery of GPR119 in animal genomes allowed for the identification of receptor ligands both *in vitro* and *in vivo*. Both endogenous and synthetic ligands have now been identified for GPR119. GPR119 has been identified in various cells in the body including the β -cells of the pancreas and the L and K cells of the intestine. The location of GPR119 has led to interest in its role in treating metabolic disorders such as Type 2 Diabetes.

The aim of this study was to examine the effect of GPR119 activation on gastrointestinal hormone and insulin secretion. GLUTag, pGIPneo STC-1 and BRIN-BD11 cells were treated with a range of concentrations (10^{-12} to 10^{-4} M) of GPR119 agonist OEA, PEA and AS1269574. The effects of this treatment on cytotoxicity was assessed via MTT assay for GLUTag and pGIPneo STC-1 cells while Alamar blue assay was used in BRIN-BD11 cells. Double immunohistochemistry was carried out both *ex vivo* and *in vitro* in order to determine the cellular localisation of GPR119 in the L and K cells of the intestine. Gene expression was further assessed using qPCR. qPCR analysis quantified the expression of GPR119 in the small intestine while double immunohistochemistry was used to determine the co-localisation of GPR119 with GLP-1, GIP and PYY. Acute *in vivo* effects of the agonists AS1269574 and OEA was studied at $0.1\mu\text{mol/kg}$ body weight in lean mice ($n=6$). The biological effects of chronic AS1269574 and AS1269574 in combination with Sitagliptin treatment was assessed in streptozotocin induced diabetic mice ($n=6$).

In GLP-1 secreting GLUTag cells PEA increased GLP-1 secretion 1.3 to 2.5-fold ($p<0.01$ - $p<0.001$) at concentrations of 10^{-8} M to 10^{-4} M while OEA (10^{-6} to 10^{-4} M), increased GLP-1 secretion 1.8 to 2.0-fold ($p<0.001$). AS1269574 increased GLP-1 secretion 1.6-fold (10^{-6} M, $p<0.01$). The secretory ability of PEA on GLUTag cells was reduced 1.3 to 1.8-fold (10^{-8} M to 10^{-4} M, $p<0.05$ to $p<0.01$) when cells were treated in combination with Exendin-9 while the secretory ability of OEA was reduced 1.2 to 2.0-fold (10^{-8} M to 10^{-4} M, $p<0.05$ - $p<0.001$). Exendin-9 reduced AS1269574 secretory action 2.0-fold at 10^{-6} M ($p<0.05$).

GPR119 agonists increased GIP secretion in pGIPneo STC-1 cells. PEA increased GIP secretion 1.1 to 1.8-fold increase (10^{-8} M to 10^{-6} M, $p<0.05$). In the presence of 10^{-7} M Exendin-9, this action was decreased 1.6-fold (10^{-6} M, $p<0.05$). OEA increased GIP secretion with a 1.9-fold at 10^{-6} M ($p<0.01$) while Exendin-9 reduced this effect 1.5-fold ($p<0.05$).

In 5.6mM glucose AS1269574 stimulated insulin secretion 1.3 to 2.4-fold (10^{-8} - 10^{-4} M, $p<0.01$ - $p<0.001$) from BRIN-BD11 cells while OEA demonstrated a 1.4 to 2.2-fold increase (10^{-8} - 10^{-4} M, $p<0.05$ - $p<0.001$). PEA augmented insulin secretion 1.1 to 1.5-fold ($p<0.05$ - $p<0.001$) at concentrations of 10^{-8} to 10^{-4} M. AS1269574 ($EC_{50} = 1.17 \times 10^{-7}$ M) was the most potent agonist tested in 5.6mM glucose followed by PEA ($EC_{50} = 1.60 \times 10^{-7}$ M) and OEA ($EC_{50} = 2.83 \times 10^{-7}$ M).

In 16.7mM glucose, AS1269574 increased insulin secretion 1.2 to 2.4-fold (10^{-8} - 10^{-4} M, $p<0.01$ - $p<0.001$) from BRIN-BD11 cells. OEA demonstrated a 1.1 to 1.7-fold (10^{-10} - 10^{-4} M, $p<0.05$ - $p<0.001$) increase while PEA demonstrated a 1.2 to 1.7-fold increase (10^{-8} - 10^{-4} M, $p<0.05$ - $p<0.001$). PEA demonstrated the highest potency ($EC_{50} = 7.7 \times 10^{-8}$ M) whilst OEA ($EC_{50} = 1.3 \times 10^{-7}$ M) demonstrated a greater potency than AS1269574 ($EC_{50} = 1.3 \times 10^{-7}$ M) in 16.7mM glucose. All agonists tested demonstrated no adverse effects on cell viability or proliferation at both concentrations of glucose tested as measured by MTT.

Immunohistochemistry in both lean and HFF mouse small intestine and GLUtag cells determined the localisation of GPR119 and GLP-1 in the L-cells of the intestine as well as GIP and GPR119 in intestinal K cells. The presence of GPR119 in the L-cells of the intestine as co-localisation was observed between GPR119 and PYY in STC-1 cells and both lean and HFF NIH Swiss mouse small intestine. Gene expression analysis of NIH Swiss mouse small intestine via qPCR showed that a HFF diet increases GPR119 expression 1.2-fold compared to lean ($p<0.05$). GPR119 OEA and PEA demonstrated no effect on GPR119 gene expression in GLUtag cells while AS1269574 demonstrated a 1.4-fold increase ($p<0.05$).

Oral administration of AS1269574 and OEA ($0.1 \mu\text{mol/kg BW}$) to fasted male Swiss TO mice resulted in acute improved glucose homeostasis. AS1269574 reduced blood glucose 45.6 and 34.8% ($p<0.001$) 15 and 30 mins post administration while AS1269574 in combination with Sitagliptin reduced blood glucose 37.6%, 56.4%, 44.6% and 38.8% at 15, 30, 60 and 90 mins respectively ($p<0.05$ - $p<0.001$). OEA administration reduced blood glucose by 40.7% and 36.2% at 30 and 60 mins ($p<0.05$ - $p<0.001$) post administration while the addition of Sitagliptin to OEA administration resulted in a 34.2% and a 51.3% reduction in blood glucose ($p<0.05$ - $p<0.001$). Administration with Exendin-9 reduced the effect of AS1269574 by 49.7% at 15 min. OEA administered in combination with Exendin-9 had reductions of 36.8% and 31.5% at 30 and 60 mins post administration ($p<0.05$ - $p<0.01$). Sitagliptin alone reduced glycaemic excursion 30.9% at 30 mins and 54.8% at 90 mins post administration. AS1269574 demonstrated a 1.6 ($p<0.05$) to 3.4-fold increase in insulin secretion ($p<0.001$) 15 and 30 mins post administration. OEA increased insulin secretion 3.4-fold 30 mins post administration ($p<0.001$). AS1269574 in combination with Sitagliptin increased insulin secretion 2.7-fold ($p<0.001$) 30 mins post administration. OEA administered in combination with Sitagliptin also demonstrated insulinotropic effects 30 mins post administration with an increase of 3.1-fold being observed ($p<0.001$). The addition of $0.1 \mu\text{mol/kg BW}$ Exendin-9 reduced the insulin secretory effect of AS1269574 and the effects of OEA were decreased 1.9-fold ($p<0.001$).

AS1269574 administered orally increased GLP-1 secretion 2.5 and 4.9-fold 15 and 30 mins post administration ($p<0.001$). OEA increased GLP-1 secretion 2.7-fold 30 mins post administration

($p < 0.001$). Overall AS1269574 increased GLP-1 secretion 1.9-fold compared to OEA ($p < 0.01$). AS1269574 increased GIP secretion 2.7-fold ($p < 0.001$) while OEA exhibited a 3.0-fold ($p < 0.001$) increase 15 mins post administration. The overall effect of OEA on GIP secretion was 1.2-fold higher than AS1269574 ($p < 0.05$).

Chronic oral treatment with AS1269574 (0.1 $\mu\text{mol/kg}$ body weight) decreased blood glucose 35.4% ($p < 0.01$) while AS1269574 combination therapy with Sitagliptin reduced blood glucose 32.3% ($p < 0.001$). Plasma insulin was increased 2.0-fold ($p < 0.001$) in mice treated with AS1269574 while mice that received AS1269574 combination therapy exhibited an increase of 1.9-fold ($p < 0.001$) compared to streptozotocin induced diabetic mice. Chronic treatment with AS1269574 and AS1269574 in combination with Sitagliptin increased plasma incretin hormone levels compared to both lean and streptozotocin induced diabetic mice treated with saline. AS1269574 monotherapy increased plasma GLP-1 levels 2.0 and 2.5-fold compared to diabetic mice after 12 and 21 days of treatment respectively ($p < 0.001$). AS1269574 combination therapy increased plasma GLP-1 levels 2.3-fold after 21 days of treatment ($p < 0.001$). AS1269574 in combination with Sitagliptin increased plasma GIP levels 2.9-fold compared to diabetic mice after 21 days of treatment ($p < 0.001$). AS1269574 monotherapy produced a 2.2-fold increase after 21 days of treatment ($p < 0.001$).

Oral glucose tolerance tests showed that AS1269574 treated mice had a 1.7-fold ($p < 0.001$) overall reduction in blood glucose when compared to streptozotocin mice. AS1269574 in combination with Sitagliptin reduced hyperglycaemia 1.9-fold ($p < 0.001$). Overall chronic AS1269574 treatment led to a 1.4-fold increase in insulin secretion ($p < 0.01$) and AS1269574 combined with Sitagliptin increased insulin secretion 1.4-fold compared to streptozotocin treated mice ($p < 0.01$). Treatment with AS1269574 and AS1269574 in combination with Sitagliptin had no effect on insulin sensitivity.

Daily oral administration of AS1269574 decreased body weight 1.2-fold ($p < 0.05$) compared to diabetic mice. AS1269574 in combination with Sitagliptin had increased lean mass with a 1.2-fold ($p < 0.05$) increase being observed compared to streptozotocin treated mice. AS1269574 treatment had a 2.1-fold ($p < 0.05$) reduction in fat mass. In mice treated with AS1269574, bone mineral density of the femur was reduced 1.1-fold ($p < 0.05$) compared to lean mice but increased 1.3-fold compared to streptozotocin induced diabetic mice treated with saline ($p < 0.001$). AS1269574 combination therapy increased bone mineral content 1.4-fold compared to diabetic mice treated with saline ($p < 0.001$).

This study determines the role of GPR119 activation in gastrointestinal hormone secretion suggesting GPR119 have an important therapeutic role in the treatment of Type 2 Diabetes and metabolic disorders.

7.2: Introduction

GPR119 was first identified via bioinformatical analysis along with 6 other receptors (Fredriksson *et al.* 2003c). This analysis mapped the human gene for GPR119 to chromosome 26 which codes for a 335 amino acid long protein. The mouse ortholog, which is the same length as the human protein, has 82% amino acid sequence homology with the human protein. An ortholog in pufferfish was found during this analysis suggesting that GPR119 was present in early vertebrates more than 450 million years ago.

Following the identification of GPR119, it was one of many receptors which were of interest to researchers looking at the activation of orphan GPCRs with a view to using them as novel therapies. Research into GPR119 then focused on its deorphanisation which was achieved when lysophosphatidylcholine was discovered as a GPR119 agonist with the ability to increase insulin secretion from the β -cells of the pancreas (Soga *et al.* 2005). This work was further supported by the discovery of OEA as an endogenous ligand for the receptor (Overton *et al.* 2006). The identification of OEA as an endogenous ligand for GPR119 allowed the same researchers to develop a novel synthetic selective ligand for GPR119 known as PSN632408. This work focused on the appetite reducing effects of GPR119 agonists which suggests a role for GPR119 activation in reducing obesity and obesity related disorders such as Type 2 Diabetes, thus supporting the earlier research which had been carried out. It also confirmed the presence of higher levels of GPR119 gene expression in the islets of the pancreas compared with whole pancreas suggesting that GPR119 may have a role in the regulation of metabolic disorders.

The action of activated GPR119 can be blocked by a number of antagonists. One such antagonist, TM43718, blocked the action of AS1269574 and AR231453 (Engelstoft *et al.* 2014). AR436352 was also identified as an antagonist against the action of OEA and both antagonists acted as inverse agonists for GPR119 in the absence of GPR119 agonists (Engelstoft *et al.* 2014). Other GPR119 agonists that have been identified include Compound 8 and the partial antagonist Compound 1 (McClure *et al.* 2011). The GLP1R antagonist Exendin 9-39 has also demonstrated the ability to antagonise GPR119 and has also been used in studies involving glucose homeostasis (Moran *et al.* 2014a, McKillop *et al.* 2016).

Research by (Soga *et al.* 2005) and (Overton *et al.* 2006) identified GPR119 as a receptor which works through the activation of adenylate cyclase leading to the production of intracellular cAMP. (Sakamoto *et al.* 2006) complimented these results by determining that GPR119 was bound to a G_{as} protein inside the cell. However, other studies have also shown that GPR119 can increase insulin secretion through the voltage gated calcium ion channels as GPR119 activation has led to an increase in intracellular calcium (Ning *et al.* 2008, Moran *et al.* 2014a).

Further research focused on the localisation of GPR119 within the islet (Sakamoto *et al.* 2006). Using immunohistochemistry it was determined that GPR119 was co-localised with PPY in the PP-cells of the islet. However, gene expression studies confirmed the presence of GPR119 in the insulin secreting MIN6 and glucagon secreting α TC1.9 cell line suggesting that GPR119 is present in both the α and β cells of the islet. The effects of GPR119 activation on insulin secretion have since been tested in BRIN-BD11 cells (Moran *et al.* 2014a), MIN6 cells (Ning *et al.* 2008), NIT-1 cells (Yoshida *et al.* 2011) and HIT-T15 cells (Oshima *et al.* 2013) as well as *in vivo* (Moran *et al.* 2014a, Oshima *et al.* 2013, Yoshida *et al.* 2011).

In order for GPR119 to be used as a target in the regulation of metabolic disorders, it is important that insulin secretion is glucose-dependent. This was tested in relation to GPR119 activation in MIN6 cells (Lan *et al.* 2012). This study determined that the increase in insulin secretion mediated by GPR119 is glucose dependent. Furthermore this study also determined that activation of GPR119 can increase GLP-1 secretion from the intestinal L-cells. This effect was found to be independent on insulin levels and was thought to be due to higher basal calcium levels in L-cells compared to β -cells. GPR119 mediated GLP-1 secretion was confirmed in insulin resistant mice (Ahlkvist, Brown & Ahren 2013) which further supports the concept that GPR119 activation can be used as a therapeutic treatment for metabolic disorders.

The presence of GPR119 in GIP secreting K-cells of the intestine has previously been determined (Parker *et al.* 2009). However, a contrasting study found no GPR119 present in GIP secreting cells in rat duodenum (Chu *et al.* 2008) using *in situ* hybridisation. Remarkably the same study found that oral gavage of GPR119 agonist AS231453 increased plasma GIP and that this effect was lost in GPR119 knockout mice. This is further supported as GLP1R knockout mice had significantly increased levels of GIP when treated orally with AS231453 (Flock *et al.* 2011). There was not such a significant increase in plasma GIP in wild type mice upon GPR119 activation which suggests that GPR119 mediated GLP-1 secretion may have an effect on GIP secretion *in vivo*.

As GPR119 has shown the ability to augment the incretin hormones, further research has looked at other intestinal hormones which regulate insulin secretion or may play a role in metabolic disorders. One such hormone is PYY. Activation of GPR119 with GSK263 was shown to have a 5-fold increase on PYY compared to placebo in humans (Nunez *et al.* 2014a). Interestingly this study found that GPR119 activation by GSK263 had no effect on GLP-1 or GIP secretion. This is further supported by work in PYY knockout mice which showed that there was no effect on PYY secretion upon GPR119 activation while orally delivered GPR119 agonists reduced blood glucose in wild type mice (Cox *et al.* 2010) suggesting that GPR119 mediates PYY secretion.

GPR119 activation has been shown to play a role in glycaemic control through various methods as mentioned above. This study identifies the effects of GPR119 activation on insulin, GLP-1, GIP

and PYY secretion both *in vitro* and *in vivo*. This was achieved using the endogenous agonists OEA and PEA and the synthetic agonist AS1269574 and a range of cell lines along with Swiss TO mice. The effects of these agonists on the genes which code for these hormones along with the expression of the receptor itself was determined by qPCR. Immunohistochemistry determined the cellular localisation in all cell lines used along with lean and HFF diet mice. Both OEA and AS1269574 were then further investigated *in vivo*.

7.3: Materials and methods

7.3.1: Materials

OEA (ab141141), PEA (ab120345) and were purchased from Abcam (Cambridge, UK). AS1269574 was purchased from R & D Systems (Minnesota, USA). Multi species GLP-1 total and rat/mouse GIP total ELISA kits were purchased from Merck Millipore (Watford, UK).

7.3.2: Acute hormone secretion from intestinal cell lines

GLUTag or pGIPneo STC-1 cells were seeded into 24-well plates with 150000 cells per well which were incubated overnight in 1ml of culture medium (as described previously in Chapter 2, section 2.1) at 37°C and 5% CO₂. To ensure that cells grew in monolayers, they were pipetted up and down vigorously to ensure proper mixing before seeding. After overnight incubation and preincubation with 1.1mM glucose for 40 mins, acute incubations were carried out at 37°C 5% CO₂ for 2 hrs in 2mM glucose in KRBB buffer supplemented with 10⁻¹² to 10⁻⁴M concentrations of AS1269574, OEA and PEA. After incubations, supernatants (950µl) were collected and frozen at -20°C until ELISA could be carried out.

7.3.3: Acute insulin secretion from pancreatic BRIN-BD11 cells

Cells were seeded into 24-well plates with 150000 BRIN-BD11 cells per well and incubated overnight in 1ml of RPMI 1640 media at 37°C and 5% CO₂. Following this incubation, cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹² to 10⁻⁴M concentrations of AS1269574, OEA and PEA with 10mM alanine as a positive control. After incubations, supernatants (950µl) were collected and frozen at -20°C until radioimmunoassay could be carried out.

7.3.4: Acute effects of GPR119 agonists on cell toxicity

Cells were seeded in 96-well plates with 40000 cells per well of BRIN-BD11, GLUTag or pGIPneo STC-1 cells and incubated overnight in cell culture media (as described in Chapter 2, section 2.1) in an atmosphere of 5% CO₂ at 37°C. After 20 hrs, BRIN-BD11 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 20 mins in 5.6mM and 16.7mM glucose in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of GPR119 agonist with 1mM H₂O₂ as a cytotoxic control (n=3). After 48hrs, GLUTag and pGIPneo STC-1 cells were preincubated with 1.1mM glucose for 40 mins with subsequent acute incubations being carried out at 37°C 5% CO₂ for 2 hrs in 2mM in KRBB buffer supplemented with 10⁻¹²M to 10⁻⁴M concentrations of GPR119 agonists with 1mM H₂O₂ as a cytotoxic control (n=3). After incubation, supernatant was decanted, cells were washed with HBSS and 0.5mg/ml MTT was added for 2 hrs. Following MTT incubation, supernatant was discarded and DMSO was added to solubilise crystals. Optical density was subsequently measured at 570nm and 630nm using the Flexstation 3 (Molecular Devices, CA, USA).

7.3.5: Immunofluorescence staining in GLUTag, pGIPneo STC-1, STC-1 cells and lean and HFF NIH Swiss mouse small intestine

GLUTag, pGIPneo STC-1 and STC-1 cells were seeded at a density of 40000 cells on to polylysine coated slides overnight at 37°C in an atmosphere of 95% air and 5% CO₂ as described in Chapter 2, section 2.1. Swiss TO mice were anaesthetised by isoflurane and killed by cervical dislocation. Pancreas and intestine was excised as described in Chapter 2, section 2.6.3. Immunohistochemistry was carried out by incubating with rabbit polyclonal anti-GPR119 and goat polyclonal anti-GLP-1, goat polyclonal anti-GIP or goat polyclonal anti-PYY with the dilutions outlined in Chapter 2, Table 2.1.

7.3.6: Quantitative real-time PCR (qPCR)

qPCR was performed using LightCycler® 480 SYBR Green I Master. Reactions were performed using 4.5µl of 2x concentrated SYBR green, 1µl of forward and reverse primers (primer sequences are listed in Chapter 2, Table 2.2), 1µl of cDNA and 3µl of RNase free water (n=3). All reactions included a negative template control with RNase free water added instead of cDNA (n=3). The reactions were carried out in 8-well real-time PCR Tube Strips and Masterclear™ Cap Strips (Eppendorf, HAM, DE). Amplification conditions were 5 mins initial denaturation at 95°C, followed by 40 cycles of 30 secs denaturation at 95°C, 30 secs annealing at 58°C and 30 secs

extension at 72°C and a final elongation step for 5 mins at 72°C. Reactions included melting curve analysis with temperature range of 60 °C to 90 °C. All reactions were carried out using the MiniOpticon two colour real time PCR detection system (BioRad, UK). Analysis of results was performed using the Livak method and the mRNA levels of all genes were normalised using the housekeeping gene GAPDH.

7.3.7: Acute *in vivo* glucose tolerance tests

Male Swiss TO mice were housed as described in Chapter 2, section 2.7. The effects GPR119 agonists AS1269574 and OEA (0.1µmol/kg BW), as well as in combination with Sitagliptin Phosphate (50mg/kg BW) in lean male Swiss TO mice were investigated. Before test compounds were administered mice were fasted for 18 hrs and blood subsequently collected (t=0). Test compounds were administered orally in glucose (18mmol/kg BW), and blood was collected at 15, 30, 60, 90 and 120 mins along with blood glucose measurements. Collected blood was centrifuged at 13200rpm for 5 mins at 4°C using a Beckman centrifuge (Beckman Instruments, UK). Plasma was stored at -20°C until hormone measurements by RIA or ELISA as described in Chapter 2, section 2.2 and 2.3.

7.3.8: Multiple low dose streptozotocin induced-diabetic mice

Age-matched (26 wks), male Swiss TO mice were sourced from Envigo (Huntingdon, UK). Mice were housed in single cages and were supplied drinking water and standard rodent maintenance diet (60% carbohydrate, 30% protein, 10% fat (12.99 kJ/g), Trouw Nutrition, Cheshire, UK) *ad libitum*. Diabetes was chemically induced in 4 hr fasted Swiss TO mice through 4 intraperitoneal injections of streptozotocin (40mg/kg body weight, 0.1M sodium citrate, pH 4.5) which were received on alternate days. Diabetes was confirmed through an oral glucose tolerance test (OGTT) which was performed 14 days after the first injection of streptozotocin injection (day 0) on 18 hr fasted Swiss TO mice.

7.3.9: Treatment procedure and parameters assessed

The long term effects of oral AS1269574 (0.1µmol/kg BW) or saline (0.9% w/v NaCl) in multiple low dose streptozotocin-induced diabetic Swiss TO mice (n=6) was assessed over a period of 21 days. Hyperglycaemia was confirmed in diabetic mice through an oral glucose tolerance test which was performed 14 days after the first streptozotocin injection (day 0) on 18 hr fasted NIH Swiss mice as previously described (Chapter 2, section 2.8.2). Fluid intake, food intake, body weight, non-

fasted blood glucose, insulin, GLP-1 and GIP concentrations were monitored every 3 days. Blood was collected via tail vein bleeding of conscious mice and was collected and analysed for blood glucose, insulin, GLP-1 and GIP as described in Chapter 2, section 2.8.4. After the testing period, glucose tolerance tests (18mmol/kg body weight) were carried out to assess glucose tolerance on 18 hr fasted streptozotocin treated mice and Swiss TO mice on standard rodent maintenance diet as outlined in Chapter 2, Section 2.8.2.

7.3.10: Insulin sensitivity

Prior to the injection of insulin, blood glucose was measure in non-fasted Swiss TO mice via tail vein bleeding using the Bayer Contour glucose meter. Bovine insulin (25U/kg) in 0.9% saline was administered using intraperitoneal injection of bovine insulin (25U/kg) and blood glucose was monitored at 30 and 60 mins post injection.

7.3.11: Dual energy X-ray absorption (DEXA)

Non-fasted Swiss TO mice were culled by cervical dislocation after being anaesthetised using isoflurane, and subsequently arranged on the specimen tray to ensure both limbs and tail were extended away from the body. In order to ensure calibration and quality control, 25 measures of the aluminium/lucite phantom (0.069g/cm², 12.0% fat) were taken using a Lunar PIXImus Dual energy X-ray absorption (DEXA) system (software version 1.4x) before any specimens were measured. DEXA scanning was performed on all carcasses as per manufacturer's guidelines. The Lunar PIXImus DEXA system measures percentage body fat as well as bone mineral density (BDM, g/cm³) and bone mineral content (BMC, g) using a fully integrated densitometer.

7.3.12: Measurement of plasma hormones

Blood was collected via tail vein bleed on conscious mice every 3 days for biochemical analysis on a variety of hormones. Blood glucose determination was performed using a Bayer Contour Next meter (Leverkusen, DE) every 3 days. Plasma insulin was quantified using insulin RIA as previously described in Chapter 2, section 2.2. Rat/mouse GIP total ELISA kit (Millipore, Watford, UK) was used to measure total GIP concentrations in mouse plasma following chronic treatment. Multi species GLP-1 total ELISA was used to measure total GLP-1 content of mouse plasma. These sandwich based ELISAs are described in detail in Chapter 2, section 2.3.

7.3.13: Assessment of plasma lipid profiles

Plasma triglyceride and HDL cholesterol concentrations were measured using an I-lab 650 clinical chemistry instrument (Instrumentation Laboratory, Warrington, UK).

7.4: Results

7.4.1: Effects of GPR119 agonists and antagonist Exendin-9 on GLP-1 secretion from intestinal GLUTag cells

The intestinal GLP-1 secreting GLUTag cell line was treated with the GPR119 agonists tested above. In order to determine the selectivity of each agonist for GPR119, cells were treated with agonist and GPR119 antagonist Exendin-9.

Of the agonists tested, PEA demonstrated the greatest GLP-1 secretory ability demonstrating a 1.3 to 2.5-fold ($p < 0.01$ - $p < 0.001$) increase compared to basal control (Figure 7.3A) at concentrations of 10^{-8} M to 10^{-4} M. When administered in combination with 10^{-7} M Exendin-9, the secretory effect of PEA was reduced 1.3 to 1.8-fold (10^{-8} M to 10^{-4} M, $p < 0.05$ to $p < 0.01$). Addition of 10^{-7} M Exendin-9 did not completely prevent PEA from increasing GLP-1 secretion, with secretion being increased 1.4 and 2.0-fold when 10^{-6} M and 10^{-4} M PEA was added in the presence of Exendin-9.

The other endogenous GPR119 agonist tested, OEA, increased GLP-1 secretion 1.8 to 2.0-fold ($p < 0.001$, Figure 7.2A). The addition of 10^{-7} M Exendin-9 along with OEA reduced its GLP-1 secretory ability 1.2 to 2.0-fold (10^{-8} M to 10^{-4} M, $p < 0.05$ - $p < 0.001$) when compared to agonist treated cells alone. As seen with PEA, the presence of Exendin-9 did not completely prevent OEA from having a secretory effect on GLUTag cells. 10^{-4} M OEA in the presence of 10^{-7} M Exendin-9 increased GLP-1 secretion 1.4-fold.

Synthetic agonist AS1269574 (Figure 7.1A) demonstrated a 1.6-fold increase in GLP-1 secretion when compared to basal control (10^{-6} M, $p < 0.01$). In the presence of Exendin-9, the secretory action of AS1269574 was reduced 2.0-fold at 10^{-6} M ($p < 0.05$).

AS1269574 was the most potent agonist which had an effect on GLP-1 secretion with an EC_{50} of 2.7×10^{-8} M, with OEA having an EC_{50} of 2.1×10^{-7} M and PEA 1.21×10^{-6} M.

7.4.2: Effects of GPR119 agonists and antagonist Exendin-9 on GIP secretion from intestinal pGIPneo STC-1 cells

In order to assess the ability of GPR119 activation in the K cells of the intestine, pGIPneo STC-1 cells were treated with GPR119 agonists. As also used in GLUTag cells, Exendin-9 determined agonist selectivity for GPR119.

AS1269574 demonstrated no secretory effect on GIP compared to 2mM basal control (Figure 7.4A). Interestingly, at the lowest AS1269574 concentration tested, 10^{-8} M, the presence of Exendin-9 reduced GIP secretion 1.1-fold ($p < 0.05$). However, endogenous GPR119 agonist PEA demonstrated GIP secretory action with a 1.1 to 1.8-fold increase (10^{-6} M to 10^{-4} M, $p < 0.05$, Figure 7.6A). In the presence of GPR119 agonist 10^{-7} M Exendin-9, this action was decreased 1.6-fold (10^{-4} M, $p < 0.05$). All agonists tested demonstrated no cytotoxic effects as measured by MTT assay (Figures 7.4B-7.6B). The other endogenous agonists tested, OEA, increased GIP secretion 1.9-fold 10^{-4} M, $p < 0.01$, Figure 7.5A). In the presence of Exendin-9 this effect was reduced 1.5-fold ($p < 0.05$).

7.4.3: Effects of GPR119 agonists on insulin secretion from pancreatic BRIN-BD11 cells

The insulinotropic ability of synthetic GPR119 agonist AS1269574 and endogenous agonists OEA and PEA in clonal BRIN-BD11 cells were tested at both normal and hyperglycaemic conditions (Figures 7.7-7.12). AS1269574 (Figure 7.7A) stimulated insulin secretion from 1.3 to 2.4-fold (10^{-8} - 10^{-4} M, $p < 0.01$ - $p < 0.001$) whilst OEA (Figure 7.9A) demonstrated a 1.4 to 2.2-fold increase in a dose dependent manner (10^{-8} - 10^{-4} M, $p < 0.05$ - $p < 0.001$). A 1.1 to 1.5-fold increase in insulin secretion from BRIN-BD11 cells was observed from BRIN-BD11 cells treated with PEA (Figure 7.11A) at concentrations of 10^{-8} to 10^{-4} M ($p < 0.05$ - $p < 0.001$).

AS1269574 (Figure 7.8A) augmented insulin secretion 1.2 to 2.4-fold (10^{-8} - 10^{-4} M, $p < 0.01$ - $p < 0.001$) in hyperglycaemic conditions whilst OEA (Figure 7.10A) demonstrated a 1.1 to 1.7-fold (10^{-10} - 10^{-4} M, $p < 0.05$ - $p < 0.001$) increase. PEA (Figure 7.12A) demonstrated a 1.2 to 1.7-fold increase (10^{-8} - 10^{-4} M, $p < 0.05$ - $p < 0.001$) in 16.7mM glucose.

The calculation of the EC_{50} value for each agonist was used as a measure of potency. In 5.6mM glucose (Figure 7.13A), all agonists tested had very similar potencies with AS1269574 having an EC_{50} of 1.17×10^{-7} M, OEA demonstrating an EC_{50} of 2.83×10^{-7} M and the EC_{50} of PEA being calculated as 1.60×10^{-7} M. However in hyperglycaemic conditions (Figure 7.13B), PEA demonstrated the highest potency (7.7×10^{-8} M) whilst OEA (1.3×10^{-7} M) demonstrated a greater potency than AS1269574 (1.3×10^{-7} M). Both AS1269574 and OEA demonstrated lower potency in 16.7mM glucose compared to 5.6mM glucose while the reverse was observed for PEA. All agonists

tested demonstrated no adverse effects on cell viability or proliferation (Figures 7.7B-7.12B) at both concentrations of glucose tested.

7.4.4: Distribution of GPR119 and GLP-1 in intestinal GLUTag cells and lean and HFF NIH Swiss mouse small intestine

Immunohistochemistry in both lean and HFF mouse small intestine and GLUTag cells determined the localisation of GPR119 and GLP-1 in the L-cells of the intestine. GLP-1 was found to be expressed mainly inside clumps of L-cells (Figure 7.14B) while its presence was confirmed in both lean and HFF NIH Swiss mice small intestine (Figure 7.15B and 7.16B). GPR119 was found to be widespread throughout GLUTag cells (Figure 7.14C). It was also found to be present alongside GLP-1 in both lean and HFF small intestinal L-cells (Figure 7.15D and 7.16D). Gene expression analysis of NIH Swiss mouse small intestine via qPCR showed that a HFF diet increases GPR119 expression 1.2-fold compared to lean ($p < 0.05$, Figure 7.17).

7.4.5: Distribution of GPR119 and GIP in intestinal pGIPneo STC-1 cells and lean and HFF NIH Swiss mouse small intestine

Similarly to intestinal L-cells, immunohistochemistry of both lean and HFF mouse small intestine (Figure 7.19 and Figure 7.20) and pGIPneo STC-1 cells (Figure 7.18) was carried out to determine the cellular localisation of GPR119 and GIP. GIP was present in the majority of pGIPneo STC-1 cells (Figure 7.18B) while GPR119 was present at lower levels (Figure 7.18C). The presence of GPR119 and GIP was confirmed in both lean and HFF NIH Swiss mouse small intestine (Figures 7.19B and 7.20B). GPR119 was confirmed to be present in the intestine of both lean and HFF mice (Figure 7.19C and 7.20C) and double immunofluorescence determined the presence of GPR119 and GIP in the same K-cells (Figure 7.19D and Figure 7.20D).

7.4.6: Distribution of GPR119 and PYY in intestinal STC-1 and lean and HFF NIH Swiss mouse small intestine

The presence of GPR119 in intestinal L-cells was proven with double immunohistochemistry for both GLP-1 and GPR119. Double staining was then carried out to determine if GPR119 is present in PYY secreting L-cells in both lean and HFF mouse small intestine. This was confirmed demonstrating that GPR119 activation may have an effect on PYY secretion (Figure 7.22 and Figure 7.23). STC-1 cells are a PYY secreting cell line and double immunohistochemistry was carried out to determine their suitability in the study of the effects of GPR119 on PYY secretion (Figure 7.21).

Both GPR119 and PYY were found throughout STC-1 cells confirming their suitability for future in vitro experiments (Figure 7.21).

7.4.7: Effects of GPR119 agonists on GPR119 gene expression in intestinal GLUTag cells

The effects of GPR119 agonist treatment on GPR119 gene expression in GLUTag cells was assessed using qPCR (Figure 7.24). Endogenous GPR119 agonists OEA and PEA demonstrated no effect on GPR119 gene expression compared to vehicle control. AS1269574 exhibited a 1.4-fold increase in GPR119 expression when compared to 2mM glucose control ($p<0.05$).

7.4.8: Acute effects of GPR119 agonists on incretin hormones in male Swiss TO mice

In order to assess the effects of GPR119 agonists on GLP-1 and GIP secretion, both OEA and AS1269574 were administered orally and secretion levels were checked after 0, 15 and 30 mins respectively. GPR119 agonist AS1269574 demonstrated the greatest increase in GLP-1 secretion with a 2.5 and 4.9-fold increase being observed 15 and 30 mins post administration ($p<0.001$, Figure 7.25A). The endogenous GPR119 agonist OEA only increased GLP-1 secretion 30 mins post administration with a 2.7-fold increase being observed ($p<0.001$). Overall AS1269574 increased GLP-1 secretion 1.9-fold compared to OEA ($p<0.01$, Figure 7.25B).

Both AS1269574 and OEA increased GIP secretion 15 mins post administration with GIP secretion returning to basal levels 30 mins post administration (Figure 7.26A). OEA demonstrated the greatest increase on GIP secretion potentiating GIP secretion 3.0-fold ($p<0.001$). GIP secretion was increased 2.7-fold when AS1269574 was administered after 15 mins ($p<0.001$). The overall effect of OEA on GIP secretion was 1.2-fold higher than AS1269574 ($p<0.05$, Figure 7.27B).

7.4.9: Acute effects of GPR119 agonists on blood glucose and insulin in male Swiss TO mice

AS1269574 and OEA (0.1 μ mol/kg BW) were orally administered to fasted male Swiss TO mice. AS1269574 reduced blood glucose at 15 and 30 mins post administration (Figure 7.27A) when compared to glucose administration alone. Glycaemic excursion was reduced 45.6 and 34.8% respectively at these time points ($p<0.001$). To ensure the specificity of AS1269574 for GPR119, AS1269574 and OEA were administered in conjunction with GPR119 antagonist Exendin-9. Administration with Exendin-9 reduced the effect of AS1269574 by 49.7% at 15 min. Both agonists were also tested along with the DPP-IV inhibitor Sitagliptin Phosphate. Sitagliptin alone reduced glycaemic excursion 30.9% at 30 mins and 54.8% at 90 mins post administration. AS1269574

administered along with Sitagliptin reduced blood glucose from 15 mins until 90 mins post administration. Glycaemic excursion was reduced 37.6%, 56.4%, 44.6% and 38.8% at 15, 30, 60 and 90 mins respectively ($p<0.05$ - $p<0.001$). No effect was observed after 120 mins.

The effects of oral administration of AS1269574 on insulin secretion were observed both 15 and 30 mins post administration (Figure 7.29A). Insulin secretion from male fasted Swiss TO mice was increased 1.6-fold 15 mins post administration ($p<0.05$) while this was increased to a 3.4-fold increase ($p<0.001$) compared to glucose control. AS1269574 in combination with Sitagliptin increased insulin secretion 2.7-fold ($p<0.001$). The addition of $0.1\mu\text{mol/kg BW}$ Exendin-9 reduced the insulin secretory effect of AS1269574, however it was not completely diminished with a 1.7-fold increase in insulin secretion being observed 30 mins post administration ($p<0.05$). Overall AS1269574 increased insulin secretion 1.5-fold ($p<0.001$, figure 7.29B) while combination of Sitagliptin and AS1269574 increased insulin secretion 1.3-fold ($p<0.001$). Compared to the effects of Sitagliptin treatment alone, AS1269574 in combination with Sitagliptin and AS1269574 treatment alone had no significant effect on insulin secretion.

Oral OEA administration reduced blood glucose by 40.7% and 36.2% at 30 and 60 mins ($p<0.05$ - $p<0.001$) post administration (Figure 7.28A). GPR119 agonist Exendin-9 reduced the effect of OEA by 36.8% and 31.5% at 30 and 60 mins post administration ($p<0.05$ - $p<0.01$). The addition of Sitagliptin to OEA administration lead to a 34.2% and a 51.3% reduction in blood glucose ($p<0.05$ - $p<0.001$).

OEA increased insulin secretion 3.4-fold 30 mins post administration ($p<0.001$, Figure 7.30A). OEA administered in combination with Sitagliptin also demonstrated insulinotropic effects 30 mins post administration with an increase of 3.1-fold being observed ($p<0.001$). The presence of Exendin-9 reduced the insulin secretory effects of OEA, with an overall decrease of 1.9-fold being observed ($p<0.001$, Figure 7.30B).

7.4.10: Acute effects of GPR119 agonists on feeding

GPR119 agonists were orally administered orally to male Swiss TO mice which were trained to eat for 3 hrs daily. GPR119 agonists were also administered in combination with Sitagliptin. AS1269574 was unable to reduce feeding over the course of 3 hrs while AS1269574 in combination with Sitagliptin reduced appetite 1.2-fold 3 hrs post administration ($p<0.05$, Figure 7.31A).

The endogenous GPR119 agonist OEA caused an initial 1.5-fold reduction in appetite ($p<0.001$, Figure 7.31B) 30 mins post administration. Although there was no reduction in feeding 60 mins post administration, appetite was reduced 1.2-fold 90, 120, 150 and 180 mins post administration ($p<0.05$ - $p<0.01$). OEA in combination with Sitagliptin did not have an initial reduction in feeding

as seen with OEA, however appetite was reduced from 90 mins post administration. OEA in combination with Sitagliptin reduced appetite 1.2-fold ($p<0.01$) 90 mins post administration with decreases in appetite of 1.2, 1.2 and 1.3-fold being observed 120, 150 and 180 mins post administration.

7.4.11: Effects of multiple low dose streptozotocin on blood glucose and plasma insulin in male Swiss TO mice

Male Swiss TO mice administered multiple low dose streptozotocin via intraperitoneal injection had their blood glucose observed for 14 days after initial injection (Figure 7.34A). Streptozotocin induced diabetes caused a 1.6-fold reduction in blood glucose 2 days after the initial streptozotocin injection ($p<0.001$). Compared to lean mice, streptozotocin treated mice had a 1.3 and a 1.7-fold increase in blood glucose compared to lean mice 7 and 11 days post initial injection ($p<0.01$ - $p<0.001$). Seven days after the initial streptozotocin injection blood glucose had not increased however 11 days post initial injection there was a 1.5-fold ($p<0.001$) compared to initial levels. Lean mice underwent no changes in blood glucose for the duration of saline injections.

In order to confirm impaired glucose regulation in streptozotocin induced diabetic mice, both lean and mice treated with multiple low dose streptozotocin were fasted for 18 hrs and underwent an oral glucose tolerance test (Figure 7.32A). Streptozotocin induced diabetic mice had increased blood glucose compared to lean mice before glucose was administered with a 1.7-fold increase being observed ($p<0.01$). Oral administration of 18mmol/kg BW glucose increased blood glucose in both lean and streptozotocin induced mice. Streptozotocin induced diabetic mice had higher blood glucose levels than lean mice with increases of 1.3, 1.6, 1.63, 1.8 and 1.9-fold at 15, 30, 60, 90 and 120 mins post administration respectively ($p<0.05$ - $p<0.001$). The overall increase in blood glucose of streptozotocin induced diabetic mice was 1.6-fold as determined by AUC (Figure 7.32B).

Non-fasting blood glucose was also monitored before treatment started with streptozotocin induced diabetic mice having increased non-fasting blood glucose compared to lean mice with a 2.2-fold ($p<0.001$) increase in being observed (Figure 7.33A). Before treatment, streptozotocin treated mice were split into groups with no significant difference in blood glucose levels (Figure 7.33B).

Mice were fasted for 4 hrs before streptozotocin injection and streptozotocin treatment resulted in a reduction in body weight (Figure 7.35A) with a 1.1-fold decrease being observed on the last day of streptozotocin injection ($p<0.01$) and a 1.1-fold decrease being observed 14 days after the first injection ($p<0.001$). Lean mice had no reduction in body weight (Figure 7.35B) while the reduction in body weight in streptozotocin treated mice did not result in significant body weight differences

with lean mice before grouping (Figure 7.36A). After grouping, streptozotocin induced diabetic mice had no significant difference in body weight before treatment (Figure 7.36B).

7.4.12: Effects of AS1269574 on non-fasting GIP and GLP-1 levels

Treatment of streptozotocin induced diabetic mice with AS1269574 and AS1269574 in combination with Sitagliptin led to increased plasma incretin hormone levels compared to both lean and streptozotocin induced diabetic mice treated with saline (Figures 7.40A and 7.41A). AS1269574 treatment alone had the greatest effect on circulating GLP-1 levels with a 2.0 and 2.5-fold increase compared to diabetic mice after 12 and 21 days of treatment respectively ($p<0.001$, Figure 7.40A). AS1269574 combination therapy increased plasma GLP-1 levels 2.3-fold after 21 days of treatment ($p<0.001$).

AS1269574 in combination with Sitagliptin increased plasma GIP levels 1.5-fold compared to diabetic mice after 12 days of treatment ($p<0.05$, Figure 7.41A). After 21 days of treatment, circulating GIP levels were increased 2.9-fold ($p<0.001$). AS1269574 treatment alone produced a 1.5-fold increase after 12 days of treatment ($p<0.01$) and a 2.2-fold increase after 21 days of treatment ($p<0.001$).

7.4.13: Effects of GPR119 agonist AS1269574 on body weight, fluid intake, non-fasting blood glucose and plasma insulin

Mice with streptozotocin induced diabetes had increased blood glucose before treatment (Figure 7.43A). AS1269574, AS1269574 with Sitagliptin and saline treated streptozotocin induced diabetic mice had increased blood glucose 6 days after oral treatment ($p<0.001$). After 9 days of oral treatment, mice treated with AS1269574 and Sitagliptin still had increased glucose compared to lean mice (AS1269574), however mice treated with AS1269574 had no significant difference compared to lean mice. After 12 days of treatment both AS1269574 and AS1269574 combination therapy treated mice had a 1.7 and 1.6-fold reduction in blood glucose compared to streptozotocin induced diabetic mice treated with 0.9% saline ($p<0.05$). These effects were maintained for the duration of the treatment with AS1269574 administration reducing blood glucose 1.8, 1.7 and 1.9-fold compared to mice treated with saline ($p<0.05$ - $p<0.01$) after 15, 18 and 21 days of treatment respectively. AS1269574 in combination with Sitagliptin reduced blood glucose 1.8, 2.1 and 1.7-fold at the same time points ($p<0.05$ - $p<0.001$).

The body weight of streptozotocin mice treated with AS1269574 was reduced after 21 days of treatment (Figure 7.37A). AS1269574 monotherapy reduced body weight 18.7% ($p<0.05$)

compared to diabetic mice treated with saline. Interestingly both AS1269574 monotherapy and combination therapy with Sitagliptin reduced appetite (Figure 7.38B). AS1269574 combination therapy reduced appetite after 15 days of treatment with reductions of 23.0%, 34.4% and 27.2% ($p<0.01$ to $p<0.001$) after 15, 18 and 21 days respectively compared to diabetic mice administered saline. AS1269574 monotherapy displayed reductions of 27.1% and 30.4% ($p<0.01$ to $p<0.001$). AS1269574 monotherapy was also the only treatment to reduce fluid intake (Figure 7.39A) with a 2.5-fold ($p<0.001$) reduction being observed after 21 days.

7.4.14: Effects of AS1269574 on glucose tolerance and insulin sensitivity

In order to assess the effects of daily oral administration of AS1269574 or AS1269574 in combination with Sitagliptin, oral glucose tolerance tests were performed after 21 days of treatment (Figures 7.44A and 7.45A). Both AS1269574 and AS1269574 combination treatment reduced blood glucose over the course of the test (Figure 7.44A). Overall, AS1269574 treated mice had a 1.7-fold overall reduction in blood glucose when compared to streptozotocin mice dosed with saline over the course of the study (Figure 7.44B, $p<0.001$). AS1269574 in combination with Sitagliptin also had a reducing effect on hyperglycaemia with an overall decrease in blood glucose of 1.9-fold being observed ($p<0.001$).

Both AS1269574 and AS1269574 in combination with Sitagliptin had an insulintropic effect compared to streptozotocin mice when administered glucose orally (Figure 7.45A). AS1269574 in combination with sitagliptin increased insulin secretion 1.4 and 1.4-fold 15 and 30 mins post administration ($p<0.05$ - $p<0.01$). AS1269574 treated mice had a 1.3-fold increase in insulin secretion compared to streptozotocin treated mice ($p<0.05$). Lean mice demonstrated increased insulin secretion compared to streptozotocin induced diabetic mice treated with saline at all time points except for 60 mins post administration ($p<0.01$ to $p<0.001$). Overall AS1269574 treatment lead to a 1.4-fold increase in glucose stimulated insulin secretion ($p<0.01$) and AS1269574 combined with Sitagliptin increased insulin secretion 1.4-fold ($p<0.01$) compared to streptozotocin treated mice (Figure 7.45B). Treatment with AS1269574 and AS1269574 in combination with Sitagliptin had no effect on insulin sensitivity (Figure 7.46).

7.4.15: Effects of AS1269574 as measured by DEXA

Streptozotocin induced diabetic mice had the highest body weight with a 1.1-fold increase compared to lean mice (Figure 7.48A, $p<0.05$). Daily oral administration of AS1269574 decreased body weight 1.2-fold ($p<0.001$) compared to diabetic mice treated with saline, with a 1.1-fold reduction being observed when compared to lean mice ($p<0.01$). Mice treated with AS1269574 combination

therapy displayed a 1.1-fold reduction in body weight compared to diabetic mice ($p<0.05$). This same treatment increased lean mass (Figure 7.48B) with AS1269574 in combination with Sitagliptin increasing lean mass 1.2-fold compared to streptozotocin treated mice ($p<0.05$). The reduction in body weight of AS1269574 mice was likely to reduced fat mass (Figure 7.49A) with this treatment reducing fat mass 2.1-fold ($p<0.05$). This resulted in a 1.4-fold decrease in body fat % (Figure 7.49B, $p<0.05$).

AS1269574 treatment had no effect on overall bone mineral density (Figure 7.50B) while streptozotocin treated mice had the highest overall bone mineral content (Figure 7.50A). The results for bone mineral content were confirmed when focusing on the femur (Figure 7.51A), however bone mineral density was reduced in diabetic mice which displayed a reduction of 1.4-fold compared to lean mice (Figure 7.51B, $p<0.001$). In mice treated with AS1269574, bone mineral density of the femur was reduced 1.1-fold ($p<0.05$) compared to lean mice but increased 1.3-fold compared to streptozotocin induced diabetic mice treated with saline ($p<0.001$). AS1269574 combination therapy increased bone mineral content 1.4-fold compared to diabetic mice treated with saline ($p<0.001$).

7.4.16: Effects of chronic AS1269574 treatment on lipid profile

Streptozotocin treatment resulted in reduced triglycerides for all treatment groups (Figure 7.52A). Saline treated diabetic mice had a 2.9-fold ($p<0.001$) reduction in triglycerides compared to lean mice. AS1269574 monotherapy and combination therapy exhibited reductions of 2.4-fold and 2.1-fold ($p<0.001$) compared to lean mice. Chronic treatment with AS1269574 had no effect on total cholesterol (Figure 7.52B) or HDL cholesterol levels (Figure 7.53B). All diabetic mice had reduced LDL levels compared to lean mice (Figure 7.53A) with saline treated mice having a 1.9-fold reduction ($p<0.001$) and AS1269574 combination therapy exhibiting a reduction of 2.1-fold ($p<0.001$). AS1269574 monotherapy reduced LDL cholesterol 3.3-fold ($p<0.001$) compared to lean mice and 1.7-fold ($p<0.001$) compared to diabetic mice treated with saline.

7.5: Discussion

Current therapies for Type 2 Diabetes include drugs such as metformin which have the ability to reduce circulating glucose levels in the blood. GPR119 has previously demonstrated the ability to increase insulin secretion and thus blood glucose (Moran *et al.* 2014a) while knockout of GPR119 has reduced GLP-1 secretion *in vivo* (Moss *et al.* 2015). As a result, attention has focused on activating GPCRs such as GPR119 in the search for better anti-diabetic therapies. Some GPR119 agonists have even made it into clinical trials (Nunez *et al.* 2014a). This study demonstrated that

GSK1292263 did not improve incretin hormone secretion or the glycaemic control of individuals with Type 2 Diabetes but did show that it increased plasma PYY levels in the same individuals. The ability of GPR119 to regulate glucose homeostasis and the lack of improved glucose homeostasis in diabetics treated with GSK1292263 mean the identification of novel GPR119 agonists which can increase incretin hormone secretion and improve glucose homeostasis are an exciting target with a view to Type 2 Diabetes therapeutics.

The effects of GPR119 activation on insulin, GLP-1 and GIP secretion *in vitro* and *in vivo* was investigated in this study. GPR119 was first described in the pancreatic polypeptide secreting cells of the pancreatic islets (Sakamoto *et al.* 2006) however further studies described the expression of GPR119 in insulin secreting β -cells (Chu *et al.* 2008). Activation of GPR119 by the agonist AS1535907 has also previously been shown to increase insulin secretion (Yoshida *et al.* 2011). Therefore activation of GPR119 by fatty acids became a novel target for those looking to identify possible new drug targets for Type 2 Diabetes (Madiraju, Poitout 2007). In this study, the insulinotropic effects of GPR119 by both synthetic and endogenous agonists was investigated in the clonal BRIN-BD11 cell line at both normal and hyperglycaemic conditions. All agonists tested increased insulin secretion at both 5.6mM and 16.7mM glucose, confirming the insulinotropic effect of GPR119 agonists which had been described previously (Moran *et al.* 2014a). In terms of insulin secretion, the synthetic GPR119 agonist AS1269574 demonstrated the greatest ability to elicit an insulin secretory response in normal conditions. OEA was the best performing endogenous agonist in normal conditions followed by PEA. In order to determine the potency of each agonist, EC₅₀ values were calculated. In normal conditions, all agonists tested had similar potencies.

In hyperglycaemic conditions, AS1269574 demonstrated the greatest insulinotropic ability whilst OEA and PEA had the same response at the highest concentration tested. However, OEA did demonstrate an insulin secretory effect at lower concentrations when compared to PEA. The determination of EC₅₀ values for each agonist showed that PEA had the greatest potency for GPR119 despite the lower insulinotropic effect and OEA also demonstrated greater potency than the synthetic agonist AS1269574.

As well as the insulin secretory effect described above, GPR119 activation has shown to have a positive effect on GLP-1 secretion from the intestine (Lan *et al.* 2012, Moss *et al.* 2015, Panaro *et al.* 2017a). In order to determine the effects of GPR119 activation on GLP-1 secretion by both endogenous and synthetic agonists, GLUTag cells were treated with AS1269574, OEA and PEA. Double immunohistochemistry for GLP-1 and GPR119 confirmed the expression of GPR119 in GLUTag cells which confirms results which have been described previously (Lan *et al.* 2012). The endogenous agonist PEA demonstrated the greatest GLP-1 secretory ability followed by endogenous agonist OEA and the synthetic agonist AS1269574. Treatment of GLUTag cells with

GPR119 agonists OEA and AS1269574 along with Exendin-9 confirmed their selectivity for GPR119. EC₅₀ values determined that AS1269574 was the most potent agonist in GLUTag cells followed by OEA and then PEA. This confirms previous work which has shown OEA increases GLP-1 secretion in GLUTag cells and mice after intraluminal administration (Lauffer, Iakoubov & Brubaker 2009). The same study demonstrated that PEA has no effect on GLP-1 secretion in GLUTag cells however this was at lower concentrations than those used in this study. The selectivity of OEA for GPR119 was also confirmed using gene knockdown. This selectivity has been confirmed in the colon of GPR119 knockout mice (Moss *et al.* 2015). AS1269574 has also previously shown the ability to increase GLP-1 secretion *in vitro* in STC-1 cells (Chepurny *et al.* 2016).

Further to the secretory tests, gene expression analysis was carried out on GLUTag cells treated with GPR119 agonists. The effects of GPR119 activation on proglucagon, PC1 and GPR119 was assessed. Treatment of GLUTag cells with endogenous GPR119 agonists OEA and PEA had no effect on GPR119 expression when compared to vehicle control. However, synthetic agonist AS1269574 upregulates GPR119 gene expression in intestinal L cells. This is similar to results demonstrated previously which showed that this agonist increased the proglucagon gene promoter activity in the same cell line (Chepurny *et al.* 2013) Need to add more here on the significance of these findings.

GPR119 has previously been shown to be present in K cells of the intestine at similar levels to that seen in L cells (Parker *et al.* 2009, Reimann, Gribble 2016) and GPR119 agonists have previously been shown to increase GIP secretion (Chu *et al.* 2008, Flock *et al.* 2011, Patel *et al.* 2014). The *in vitro* effects of GPR119 activation on GIP secretion was determined through treatment of pGIPneo STC-1 cells with GPR119 agonists. As with GLUTag cells, double immunohistochemistry was first carried out to ensure the presence of both GIP and GPR119. AS1269574 demonstrated no ability to increase GIP secretion *in vitro*. However, endogenous GPR119 agonists OEA and PEA stimulated GIP secretion. Treatment of pGIPneo STC-1 cells with PEA and GPR119 agonist Exendin-9 confirmed the selectivity of PEA for GPR119.

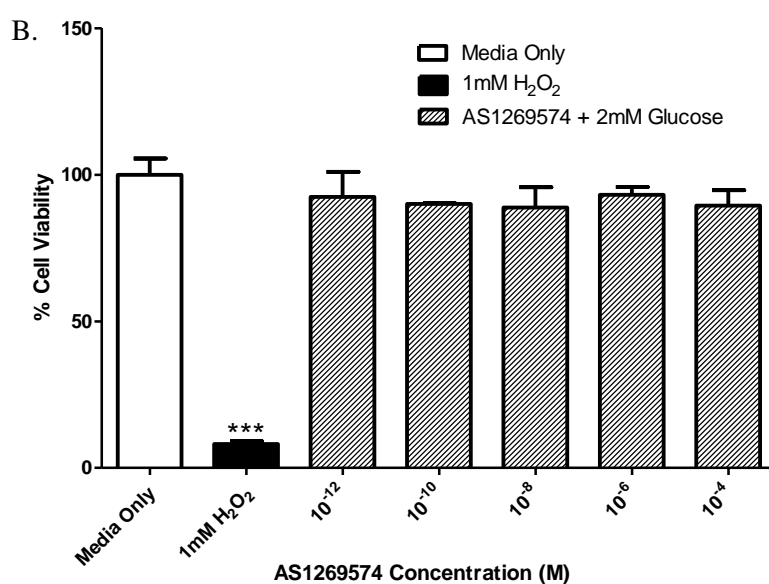
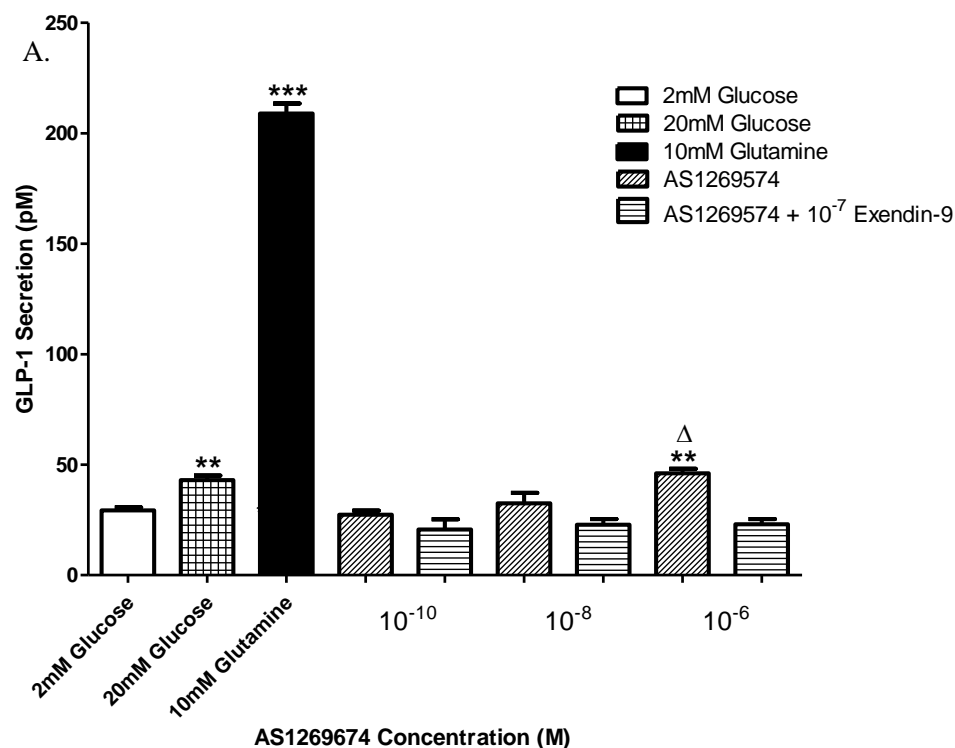
To complement the *in vitro* studies, mice were administered with either OEA or AS1269574 orally. Oral administration was chosen in order to further study the effects of GPR119 activation on intestinal hormone secretion. These agonists have been administered previously via intraperitoneal injection to confirm their insulinotropic and glucose regulatory effects (Moran *et al.* 2014a). Further work using incretin receptor knockout mice suggested a role for AS1269574 in GLP-1 secretion as *Gipr* knockout mice retained their insulinotropic ability while *Glp1r* knockout mice did not (McKillop *et al.* 2016). In this study acute male fasted Swiss TO mice received 0.1 µmol/kg body weight gavage of either AS1269574 or OEA. AS1269574 demonstrated a greater effect at reducing

blood glucose compared to OEA. However the effects of OEA were observed later than those seen with AS1269574. In order to preserve and/or enhance the incretin effect, both agonists were also tested along with the DPP-IV inhibitor Sitagliptin Phosphate. AS1269574 administered along with Sitagliptin had a more pronounced and longer lasting effect than OEA and Sitagliptin combined. Both agonists tested increased incretin hormone secretion with AS1269574 having a greater effect on GLP-1 secretion than OEA while OEA had a greater effect on GIP secretion. This confirms previous work demonstrating that AS1269574 may have an effect *in vivo* on GLP-1 secretion (McKillop *et al.* 2016). Interestingly AS1269574 also increased GIP secretion *in vivo* despite having no effect *in vitro*. This is also the first study to investigate these effects as a combination therapy with Sitagliptin.

For further confirmation of the effects displayed acutely *in vivo*, AS1269574 and AS1269574 in combination with Sitagliptin were administered chronically to mice induced with diabetes through multiple low doses of streptozotocin. This diabetic animal model exhibits moderate hyperglycaemia, hypoglycaemia and moderate levels of insulin secreting cells. Mice treated with streptozotocin demonstrated increased non-fasting hyperglycaemia, food intake, fluid intake, hyperglycaemia when challenged with glucose and body weight. Insulin secretion in response to glucose, plasma insulin, insulin sensitivity and femur bone mineral density were all reduced. Daily treatment with AS1269574 decreased non-fasting hyperglycaemia, body weight, food intake, fluid intake and body fat. Plasma insulin, plasma GLP-1, plasma GIP, femur bone mineral density and insulin secretion in response to glucose challenge were all increased. AS1269574 combination therapy decreased non-fasting blood glucose, food intake, hyperglycaemia in response to glucose and body weight were all decreased. Plasma insulin, femur bone mineral density, lean mass, insulin response to glucose challenge, plasma GLP-1 and plasma GIP were all increased with this treatment. The results described in this study confirm results seen previously for AS1269574 (Moran *et al.* 2014a, McKillop *et al.* 2016). This is the first study to describe the effects of these treatments on incretin hormones as well as the combination therapy of AS1269574 with Sitagliptin. Further studies using GPR119 knockout mice are required to further confirm the selectivity of AS1269574 for GPR119. These results also need to be studied in other diabetic animal models such as high fat fed animals.

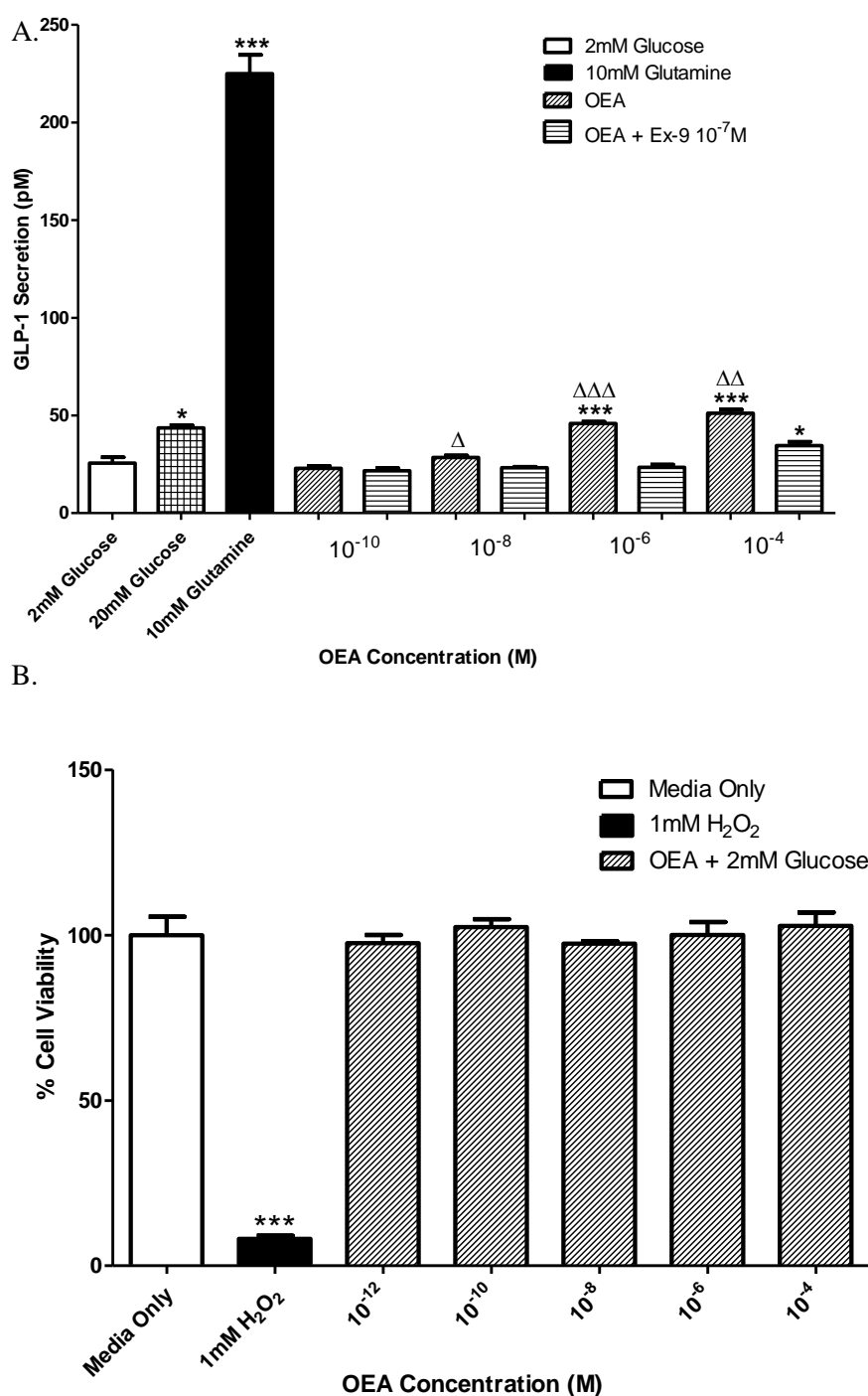
In conclusion, the insulintropic effects of GPR119 agonists in BRIN-BD11 cells were confirmed. Swiss TO mice administered GPR119 agonists orally had improved glucose homeostasis both acutely in lean mice and chronically in a Type 2 Diabetic animal model. GPR119 agonists demonstrated an ability to increase incretin hormone *in vitro* from GLUTag and pGIPneo STC-1 cells as well as acute and chronic studies *in vivo*. These findings provide further credence towards the gathering evidence of the potential of fatty acid GPCR activation as a therapy for Type 2 Diabetes.

Figure 7.1: Effects of AS1269574 on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



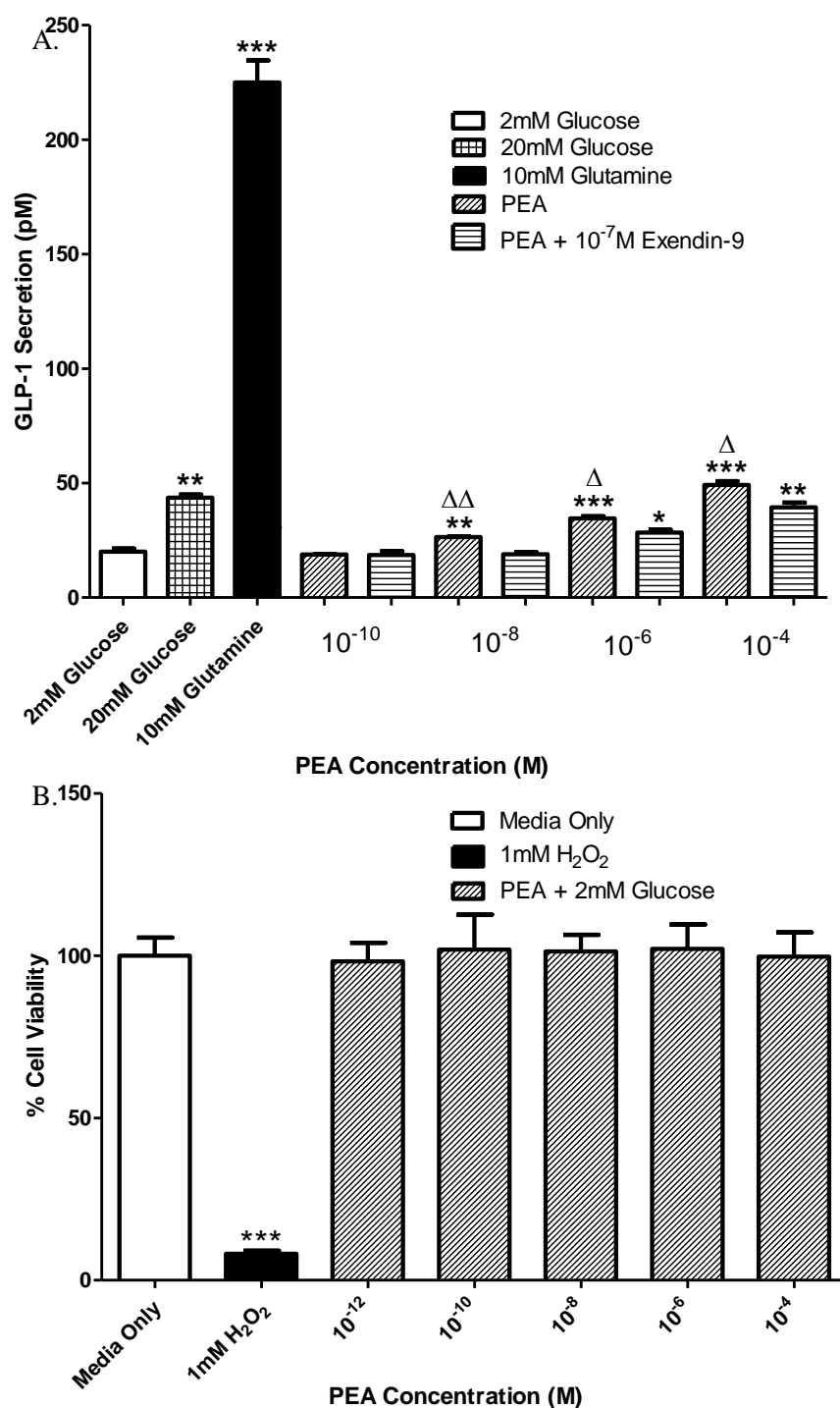
Effect of GPR119 agonist AS1269574 (10^{-10} - 10^{-4} M) and GPR119 antagonist Exendin-9 (10^{-7} M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GLP-1 secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ $p < 0.05$ compared to AS1269574 treatment alone.

Figure 7.2: Effects of OEA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



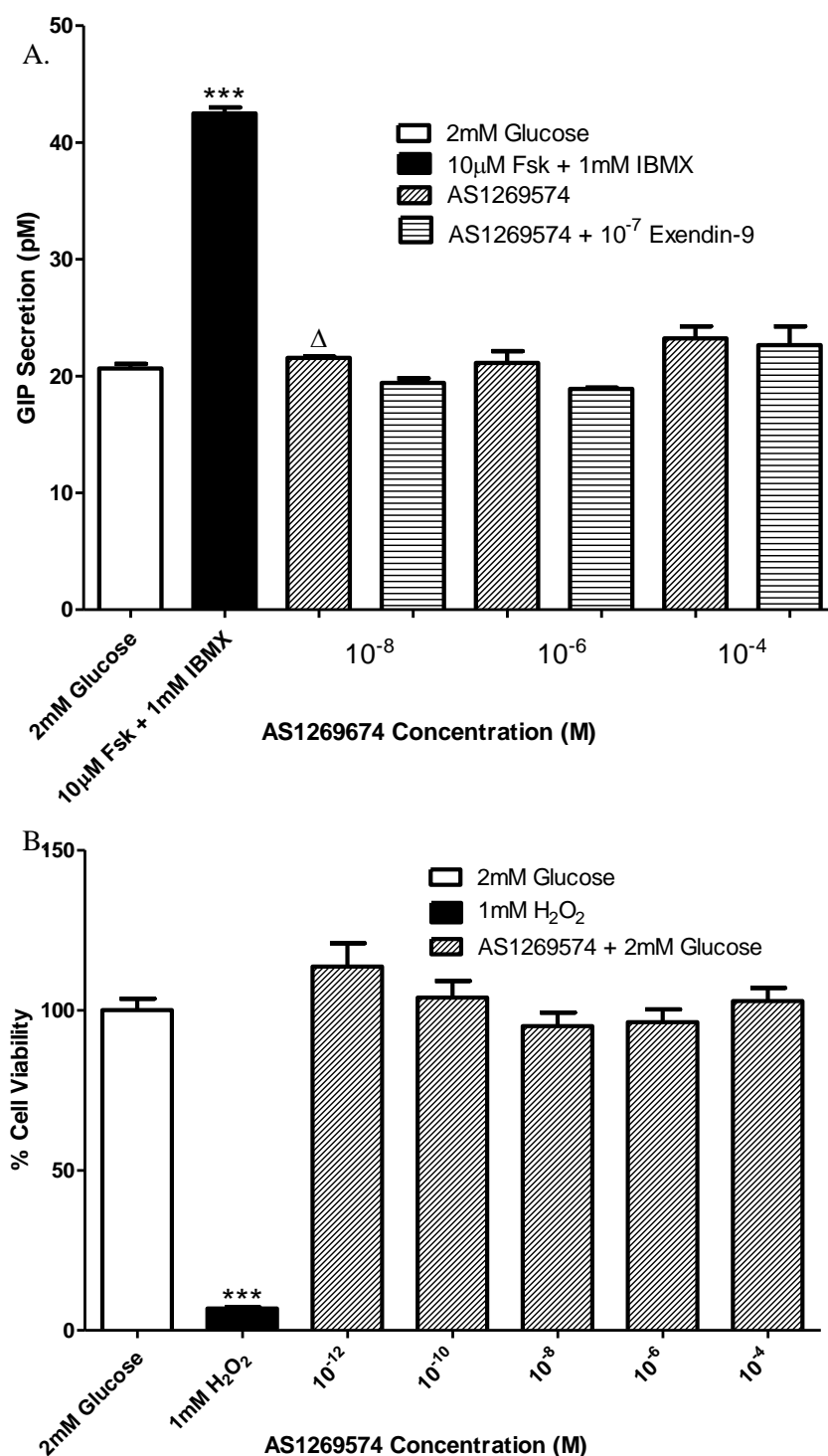
Effect of GPR119 agonist OEA (10⁻¹⁰-10⁻⁴M) and GPR119 antagonist Exendin-9 on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean ± SEM (n=3) for GLP-1 secretion and cell viability. * p<0.05, ** p<0.01 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to OEA treatment alone.

Figure 7.3: Effects of PEA on GLP-1 secretion and cell viability in intestinal GLUTag cells in 2mM glucose



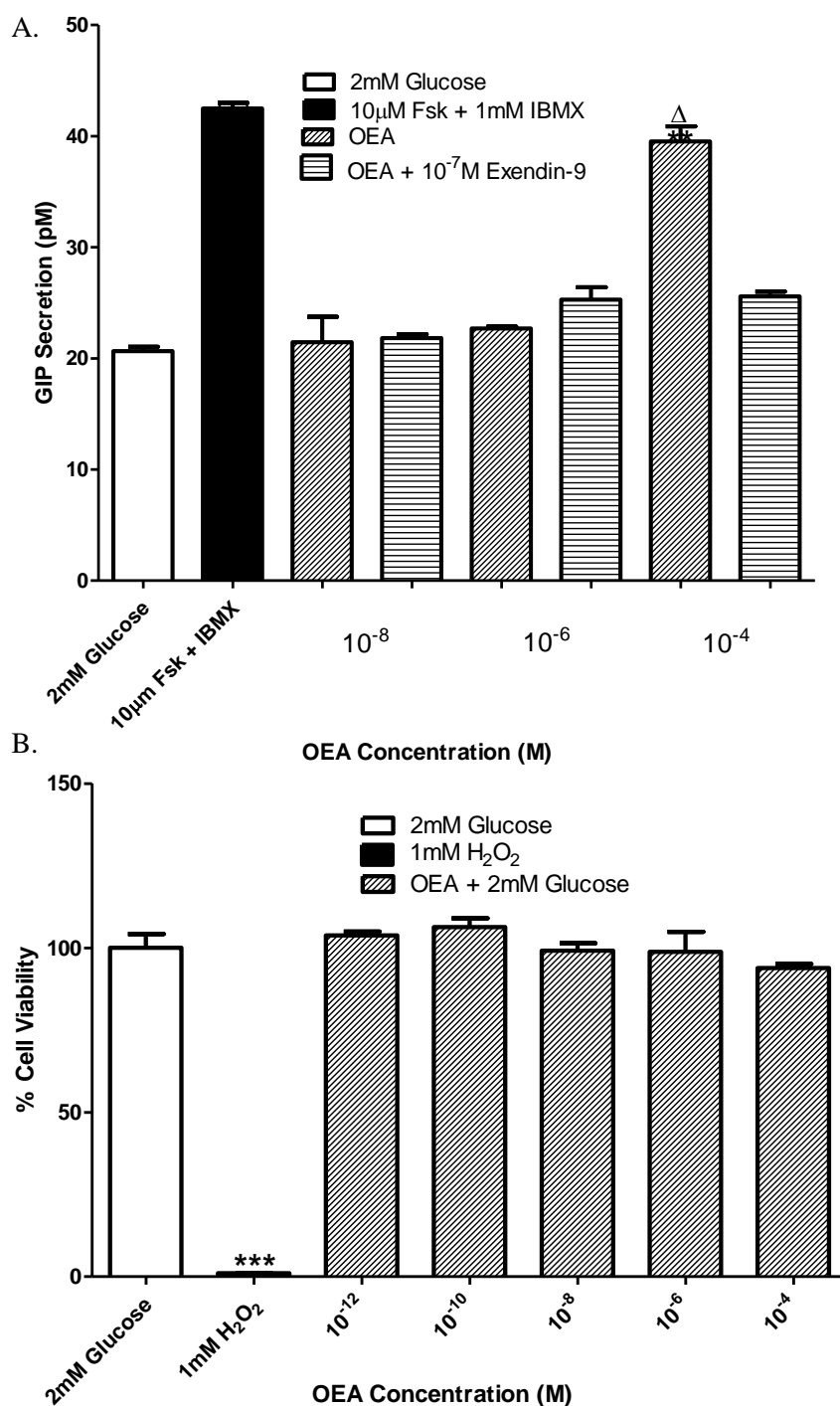
Effect of GPR119 agonist PEA (10⁻¹⁰-10⁻⁴M) and GPR119 antagonist Exendin-9 (10⁻⁷M) on GLP-1 secretion (A) and cell viability (B) at 2mM glucose in GLUTag cells. Cell viability was measured using MTT. Results are mean ± SEM (n=3) for GLP-1 secretion and cell viability. * p<0.05, ** p<0.01 and *** p<0.001 compared to basal glucose control for GLP-1 secretion and media non-toxic control for cell viability. Δ p<0.05 and ΔΔ p<0.01 compared to PEA treatment with Exendin-9.

Figure 7.4: Effects of AS1269574 on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



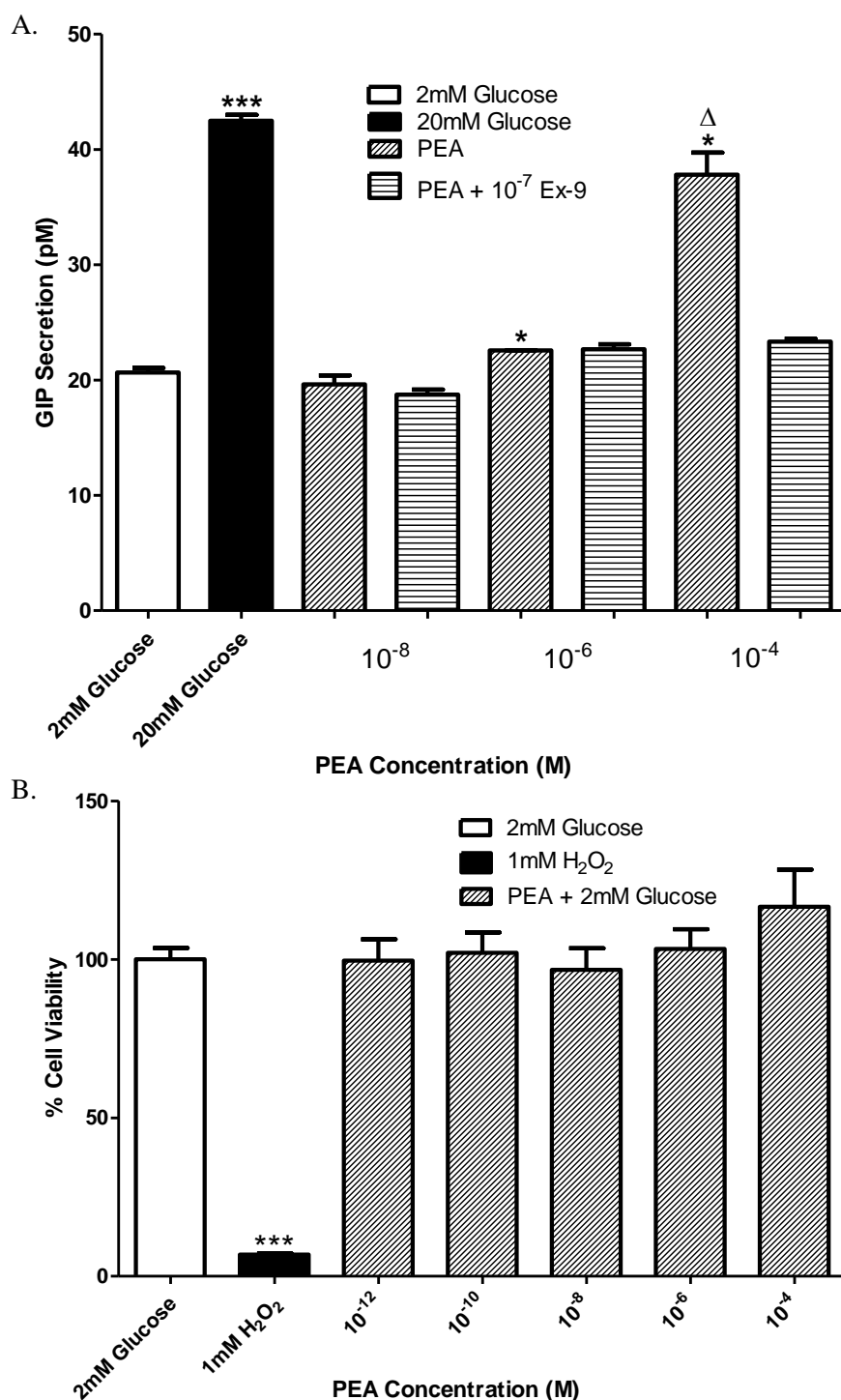
Effect of GPR119 agonist AS1269574 (10⁻¹⁰-10⁻⁴M) and GPR119 antagonist Exendin-9 (10⁻⁷M) on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean ± SEM (n=3) for GIP secretion and cell viability. *** p<0.001 compared to basal glucose control for GIP secretion and media non-toxic control for cell viability. Δ p<0.05 compared to AS1269574 treatment with Exendin-9.

Figure 7.5: Effects of OEA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



Effect of GPR119 agonist OEA (10⁻¹²-10⁻⁴M) and GPR119 antagonist Exendin-9 (10⁻⁷M) on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * p<0.05, ** p<0.01 and *** p<0.001 compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

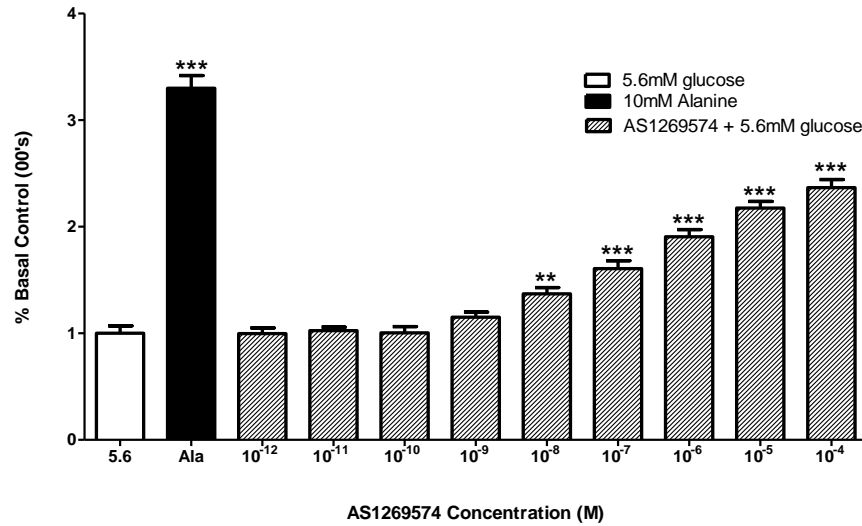
Figure 7.6: Effects of PEA on GIP secretion and cell viability in intestinal pGIPneo STC-1 cells in 2mM glucose



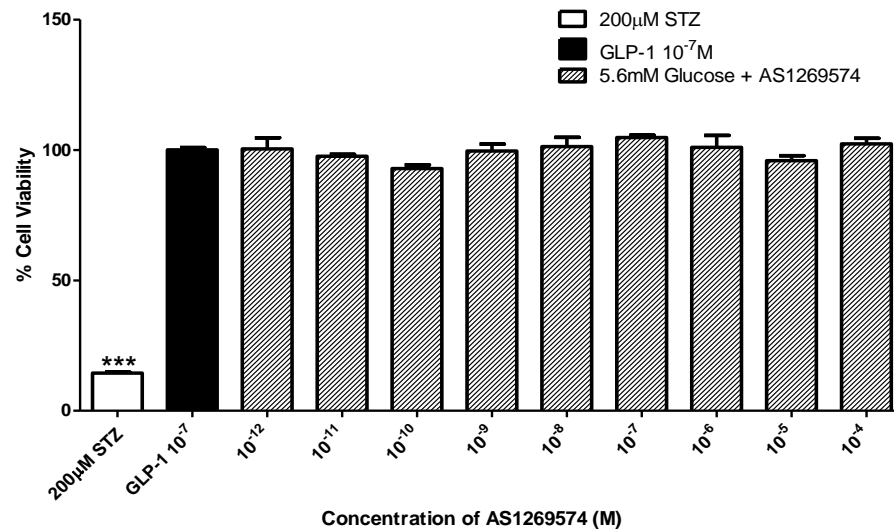
Effect of GPR119 agonist PEA (10^{-12} - 10^{-4} M) and GPR119 antagonist Exendin-9 (10^{-7} M) on GIP secretion (A) and cell viability (B) at 2mM glucose in pGIPneo STC-1 cells. Cell viability was measured using MTT. Results are mean \pm SEM (n=3) for GIP secretion and cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for GIP secretion and media non-toxic control for cell viability.

Figure 7.7: Effects of AS1269574 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.



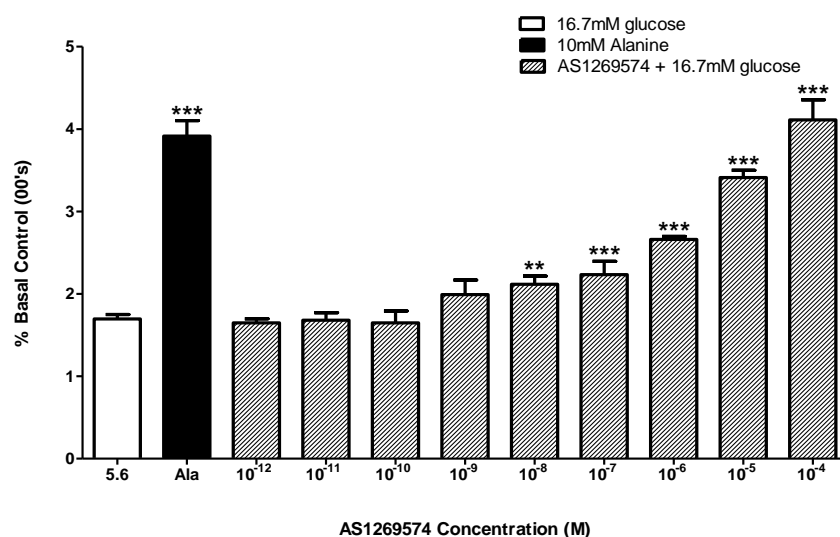
B.



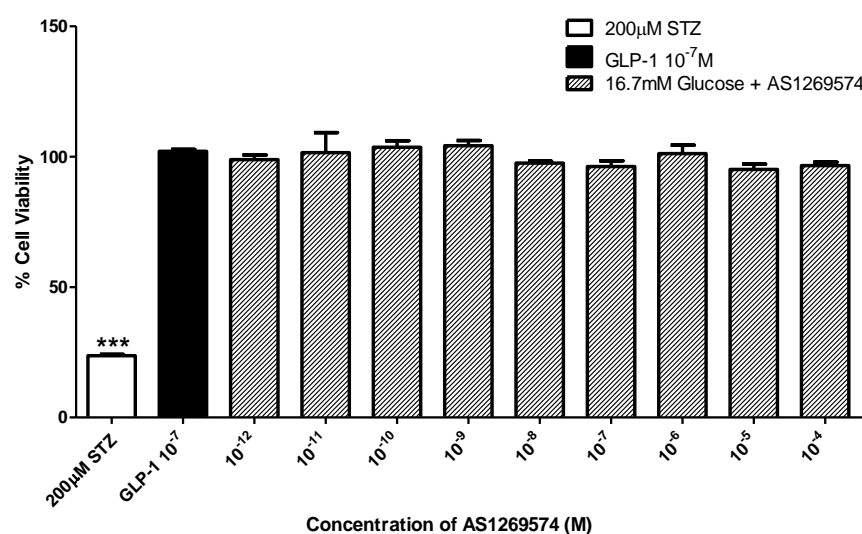
Effect of AS1269574 (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 7.8: Effects of AS1269574 on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose

A.



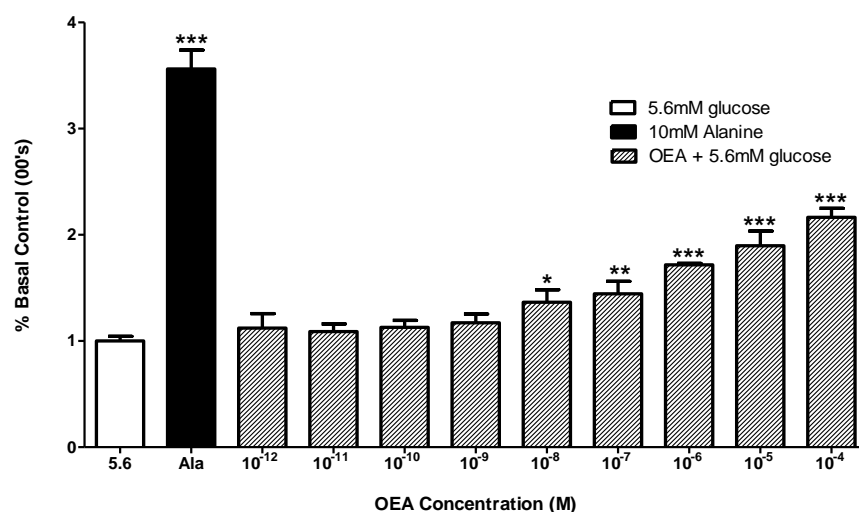
B.



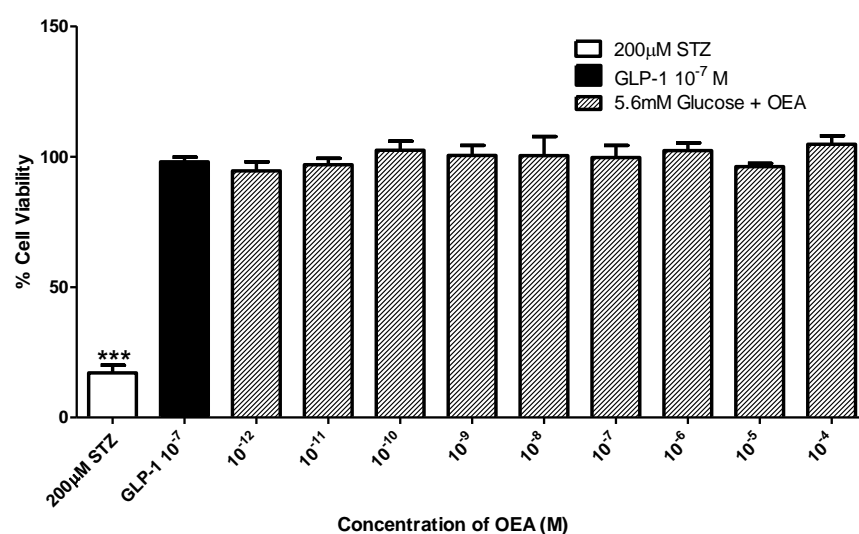
Effect of AS1269574 (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. **p<0.01 and *** p<0.001 compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 7.9: Effects of OEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose

A.

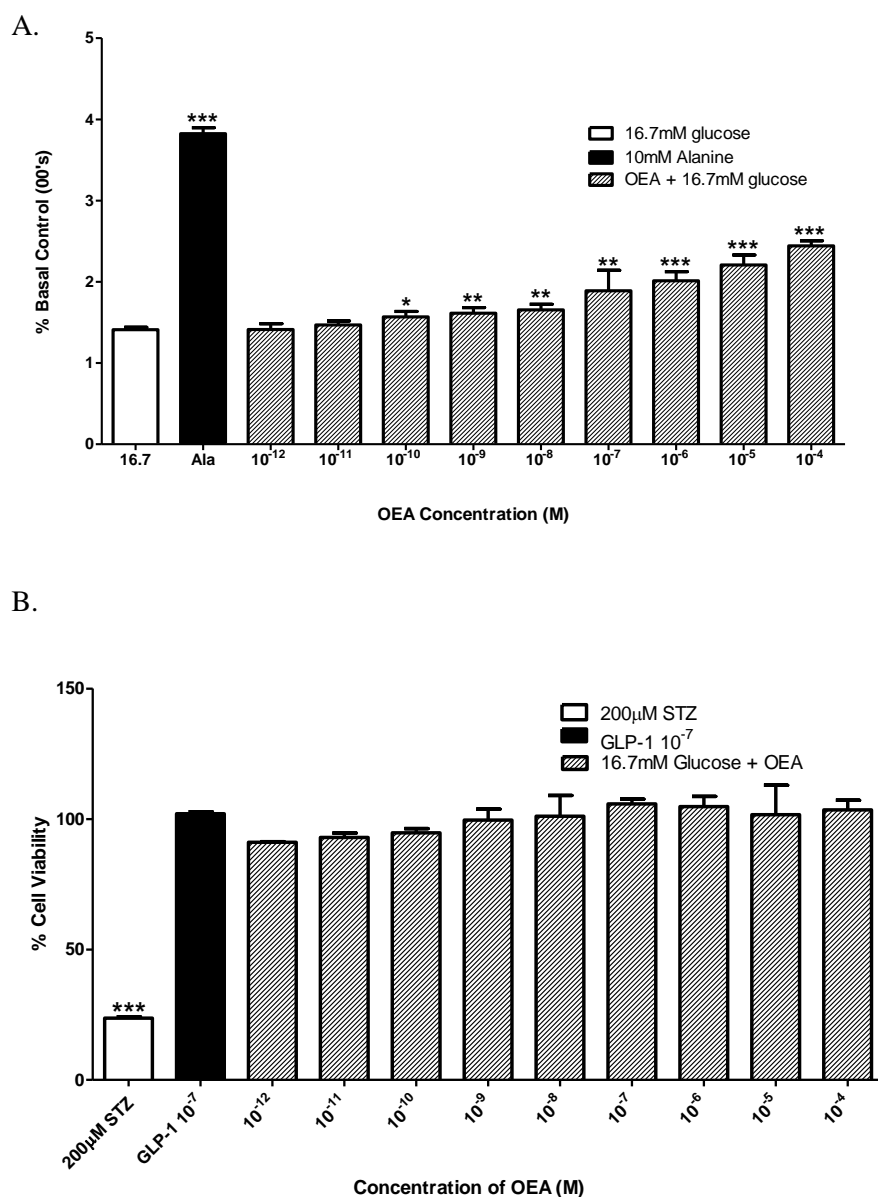


B.



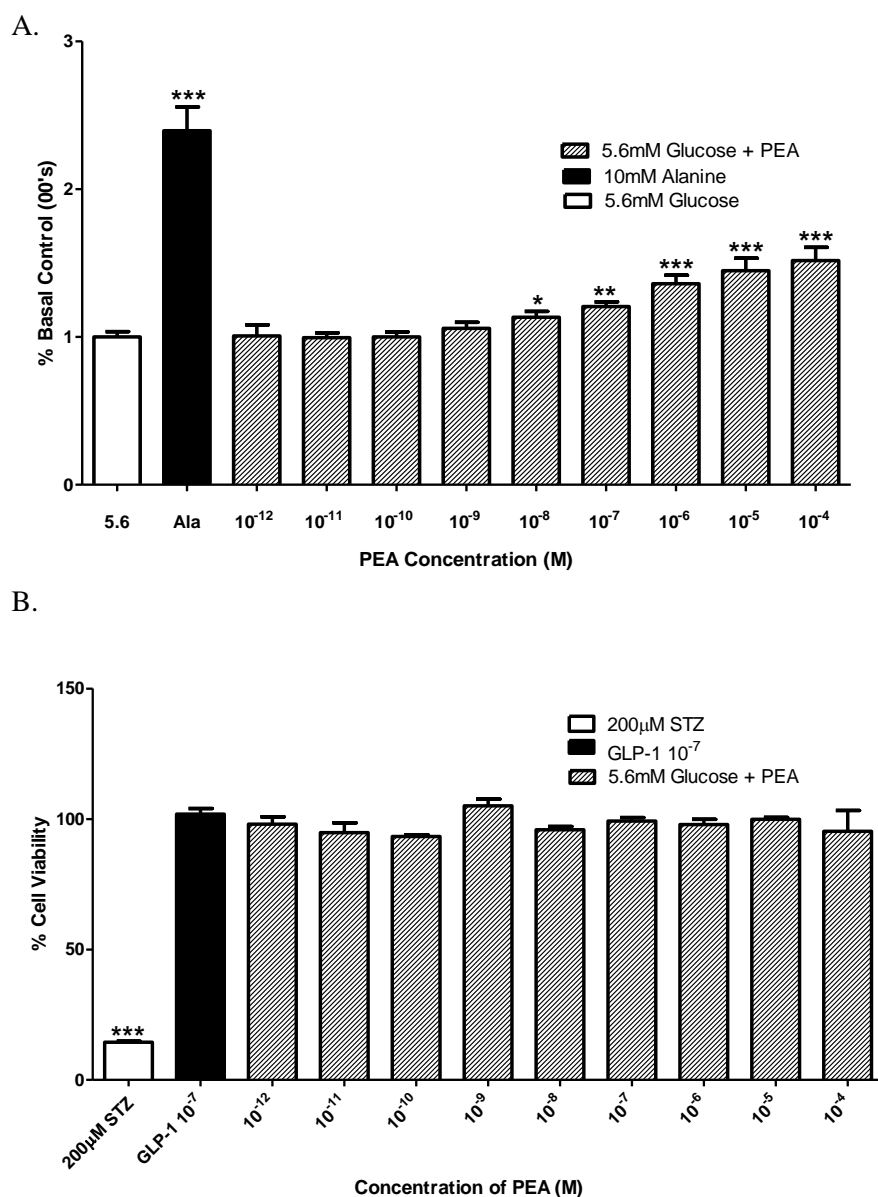
Effect of OEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 7.10: Effects of OEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



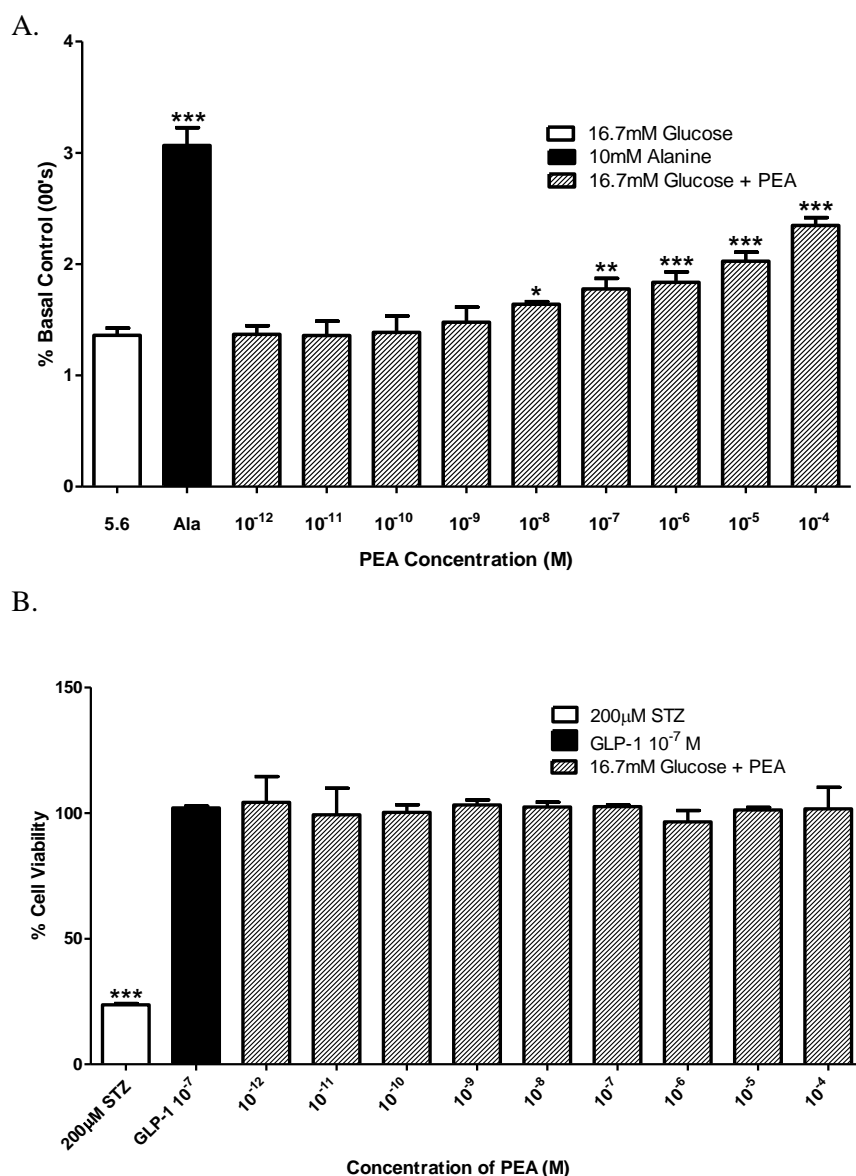
Effect of OEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 7.11: Effects of PEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 5.6mM glucose



Effect of PEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 5.6mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

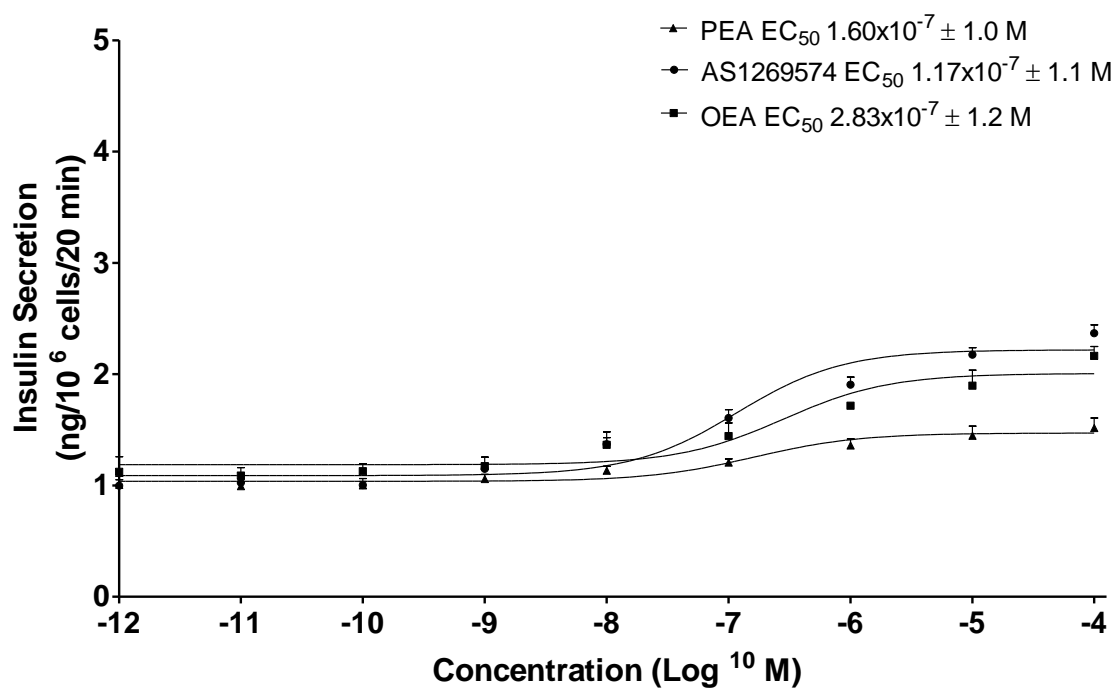
Figure 7.12: Effects of PEA on insulin secretion and cell viability in pancreatic BRIN-BD11 cells in 16.7mM glucose



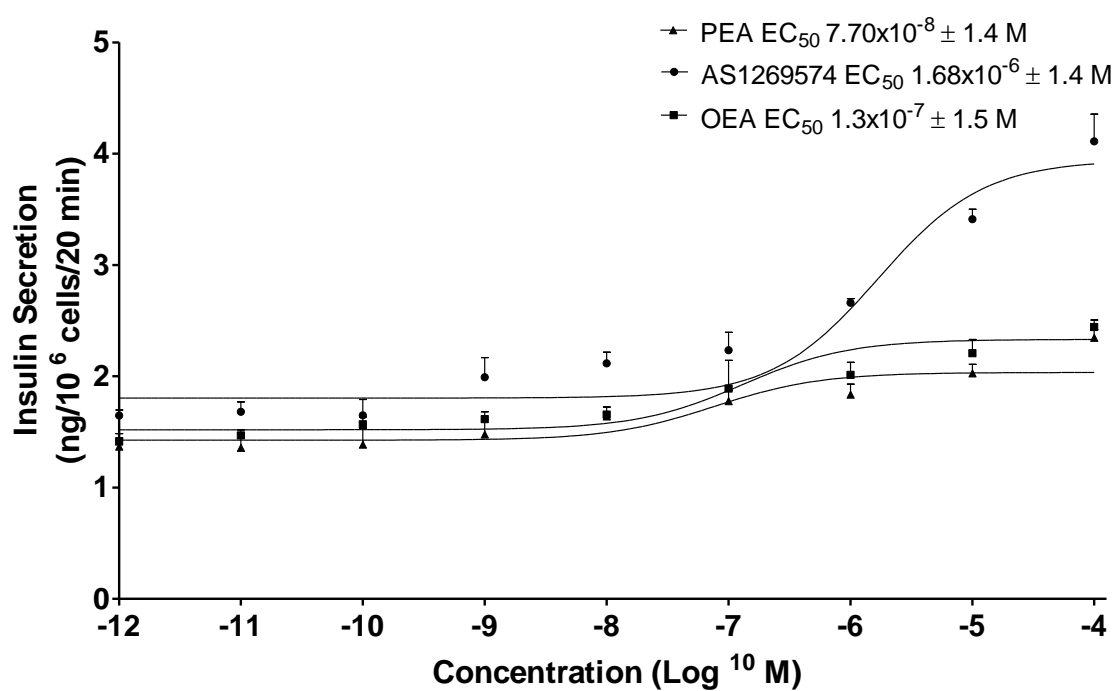
Effect of PEA (10^{-12} - 10^{-4} M) on insulin secretion (A) and cell viability (B) at 16.7mM glucose in BRIN-BD11 cells. Cell viability was measured using Alamar Blue Assay. Results are mean \pm SEM (n=8) for insulin secretion and (n=3) for cell viability. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to basal glucose control for insulin secretion and GLP-1 (10^{-7} M) non-toxic control for cell viability.

Figure 7.13: EC₅₀ of GPR119 agonists AS1269574, OEA and PEA in BRIN-BD11 cells

A.

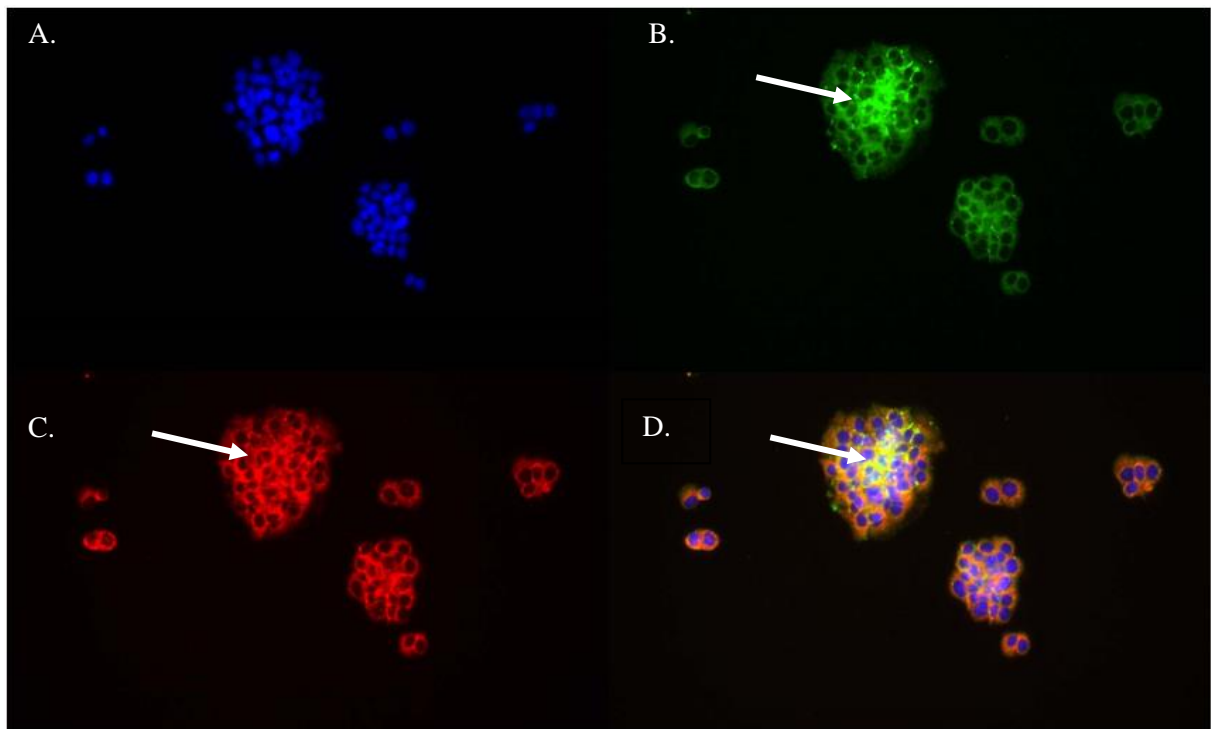


B.



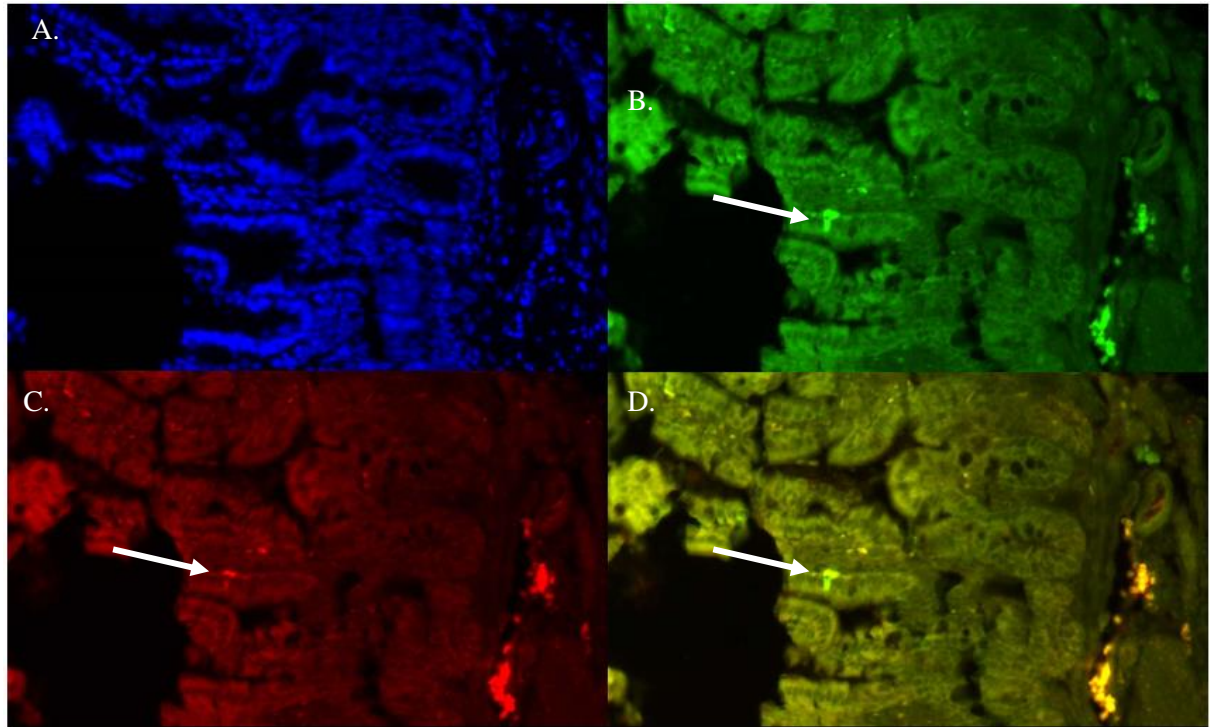
EC₅₀ values for GPR55 agonists AS1269574, OEA and PEA in BRIN-BD11 cells in 5.6mM glucose (A) and 16.7mM glucose (B).

Figure 7.14: Immunofluorescence staining of GLP-1 and GPR119 in intestinal GLUTag cells



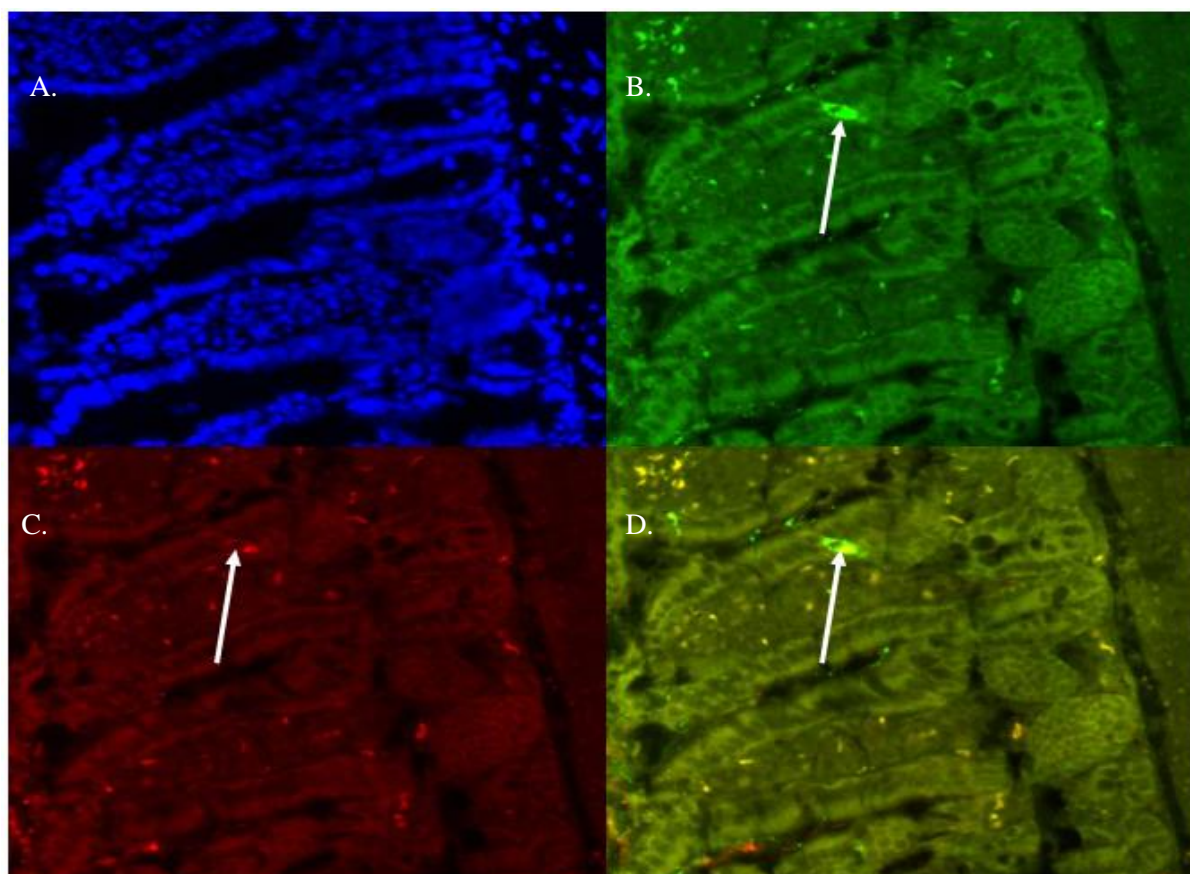
Distribution of (A) DAPI, (B) GLP-1, (C) GPR119 and (D) combined fluorescence of GLP-1 and GPR119 in GLUTag cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 7.15: Immunofluorescence staining of GLP-1 and GPR119 in lean NIH Swiss mouse small intestine



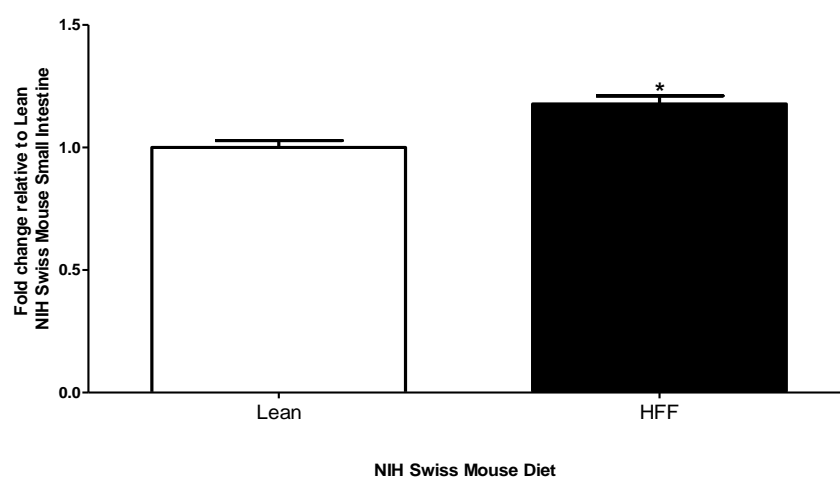
Distribution of (A) DAPI, (B) GLP-1, (C) GPR119 and (D) combined fluorescence of GLP-1 and GPR119 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 7.16: Immunofluorescence staining of GLP-1 and GPR119 in HFF NIH Swiss mouse small intestine



Distribution of (A) DAPI, (B) GLP-1, (C) GPR119 and (D) combined fluorescence of GLP-1 and GPR119 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

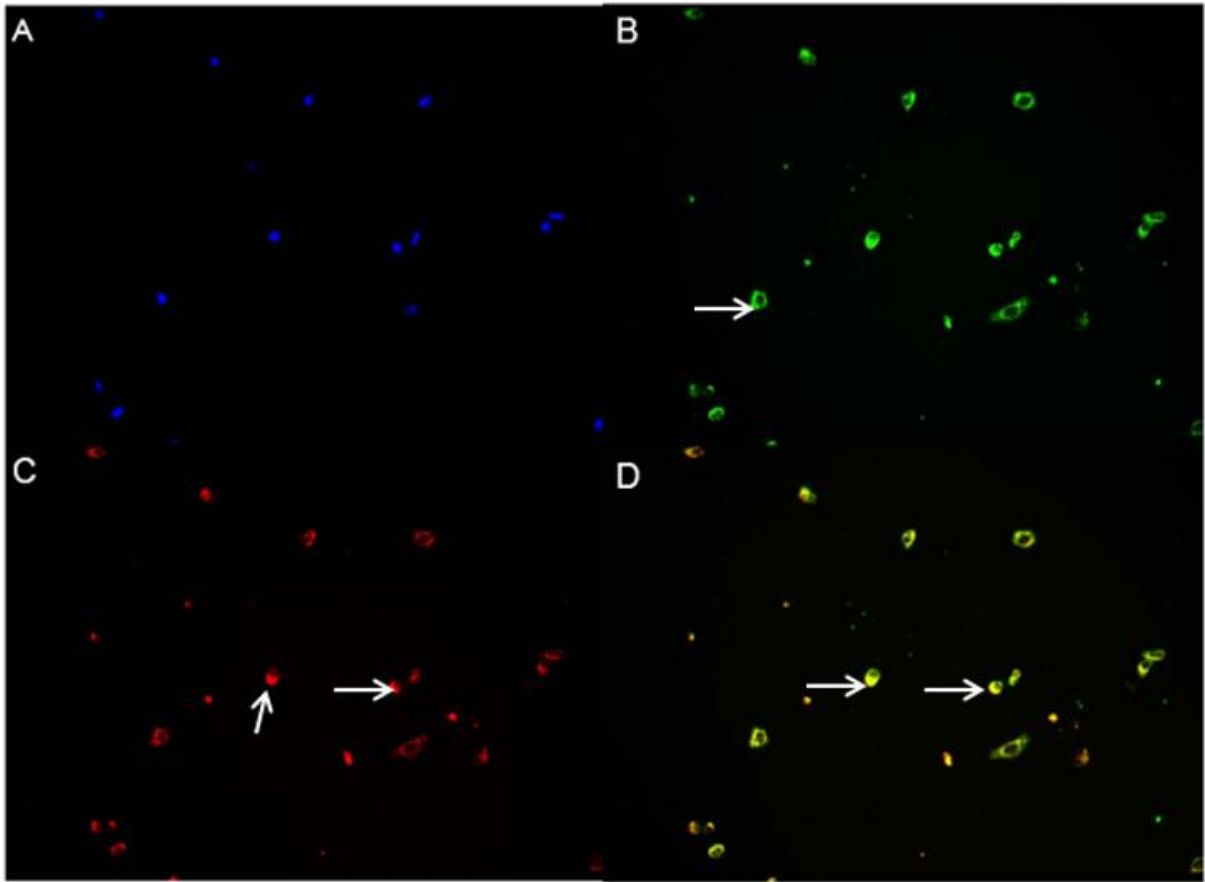
Figure 7.17: GPR119 gene expression in lean and HFF NIH Swiss mouse small intestine



Effect of HFF diet on GPR119 gene expression in male NIH Swiss mouse small intestine (n=6).

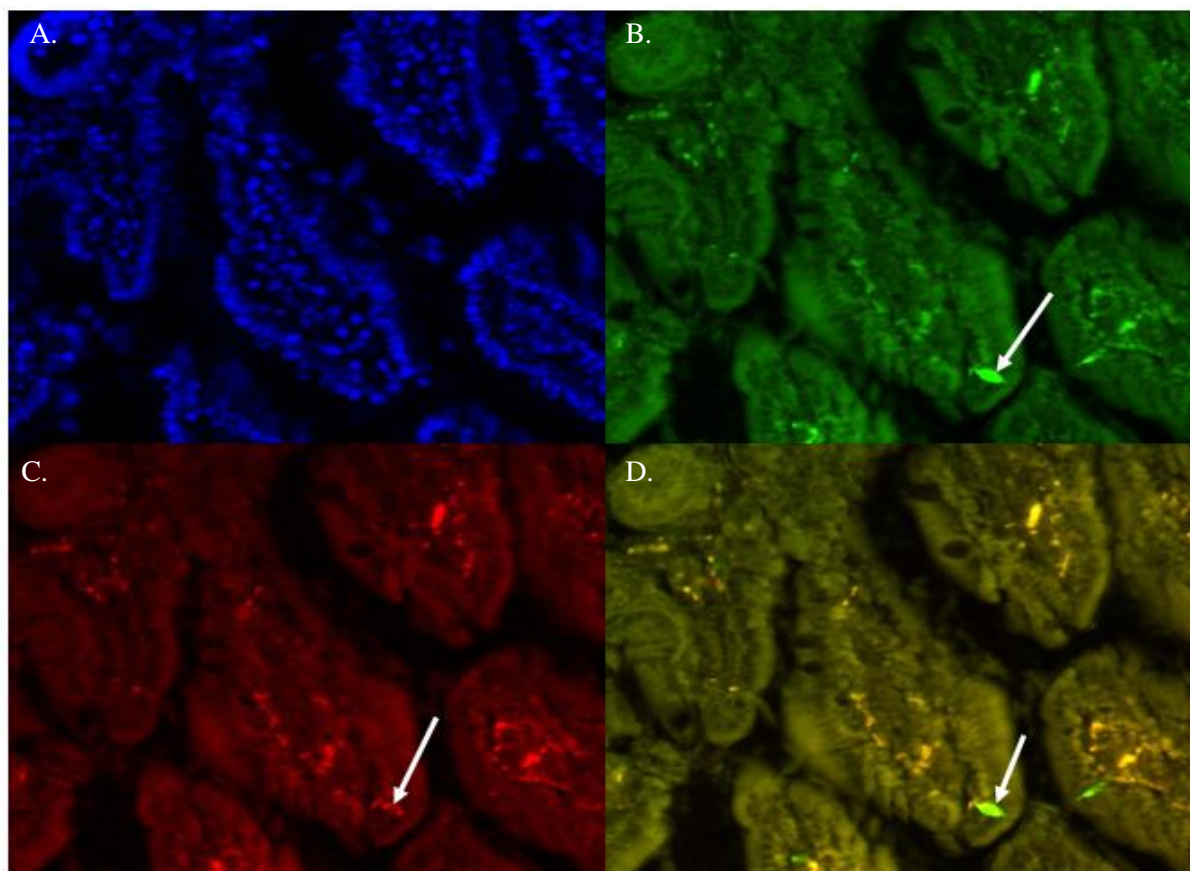
* $p < 0.05$ compared to lean mice.

Figure 7.18: Immunofluorescence staining of GIP and GPR119 in pGIPneo STC-1 cells



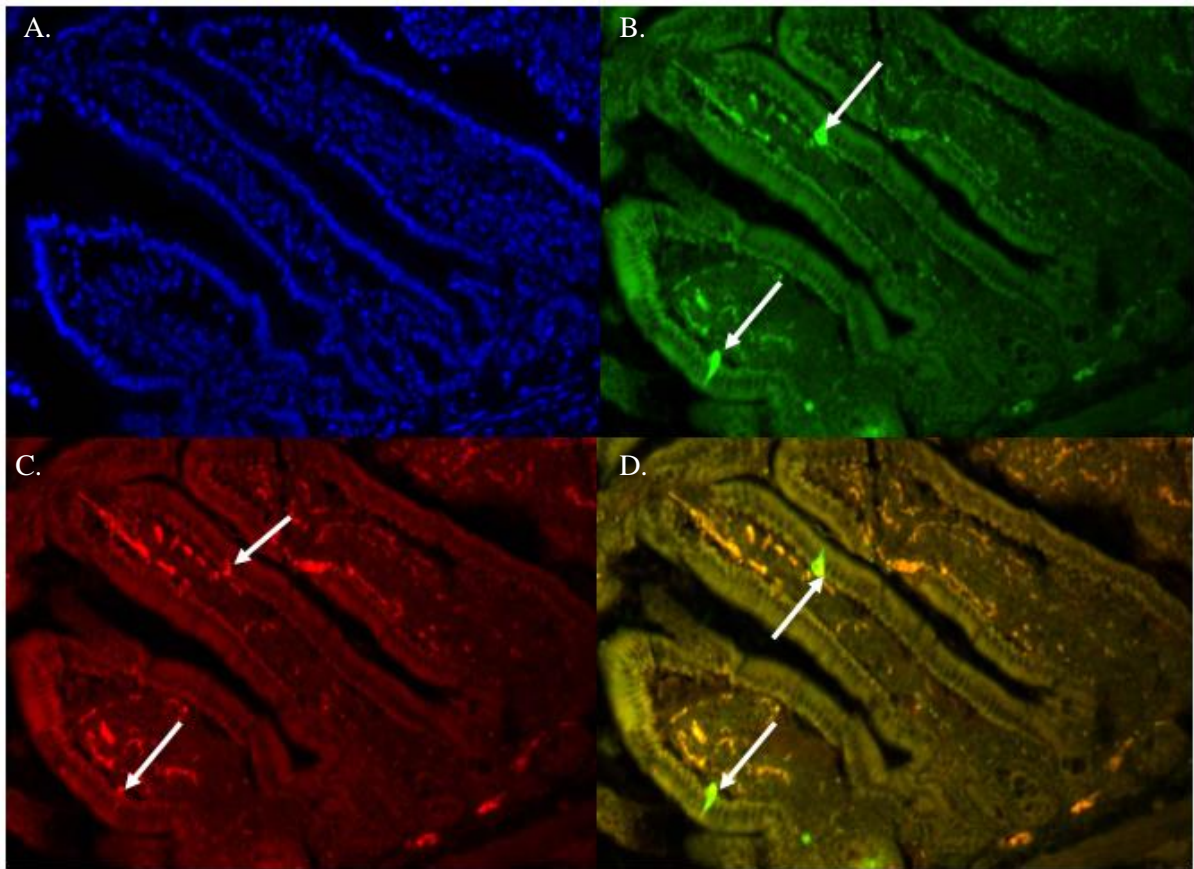
Distribution of (A) DAPI, (B) GIP, (C) GPR119 and (D) combined fluorescence of GIP and GPR119 in pGIPneo STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 7.19: Immunofluorescence staining of GIP and GPR119 in lean NIH Swiss mouse small intestine



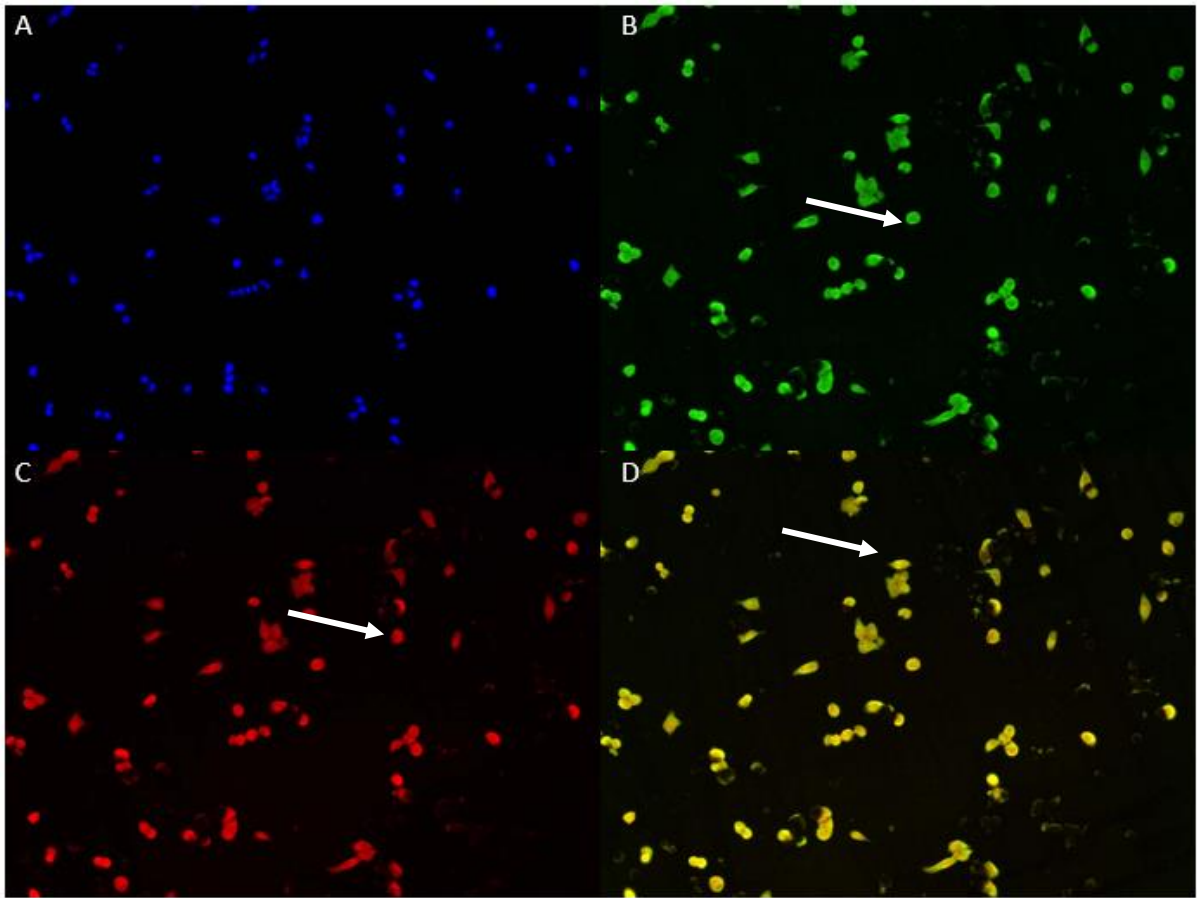
Distribution of (A) DAPI, (B) GIP, (C) GPR119 and (D) combined fluorescence of GIP and GPR119 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 7.20: Immunofluorescence staining of GIP and GPR119 in HFF NIH Swiss mouse small intestine



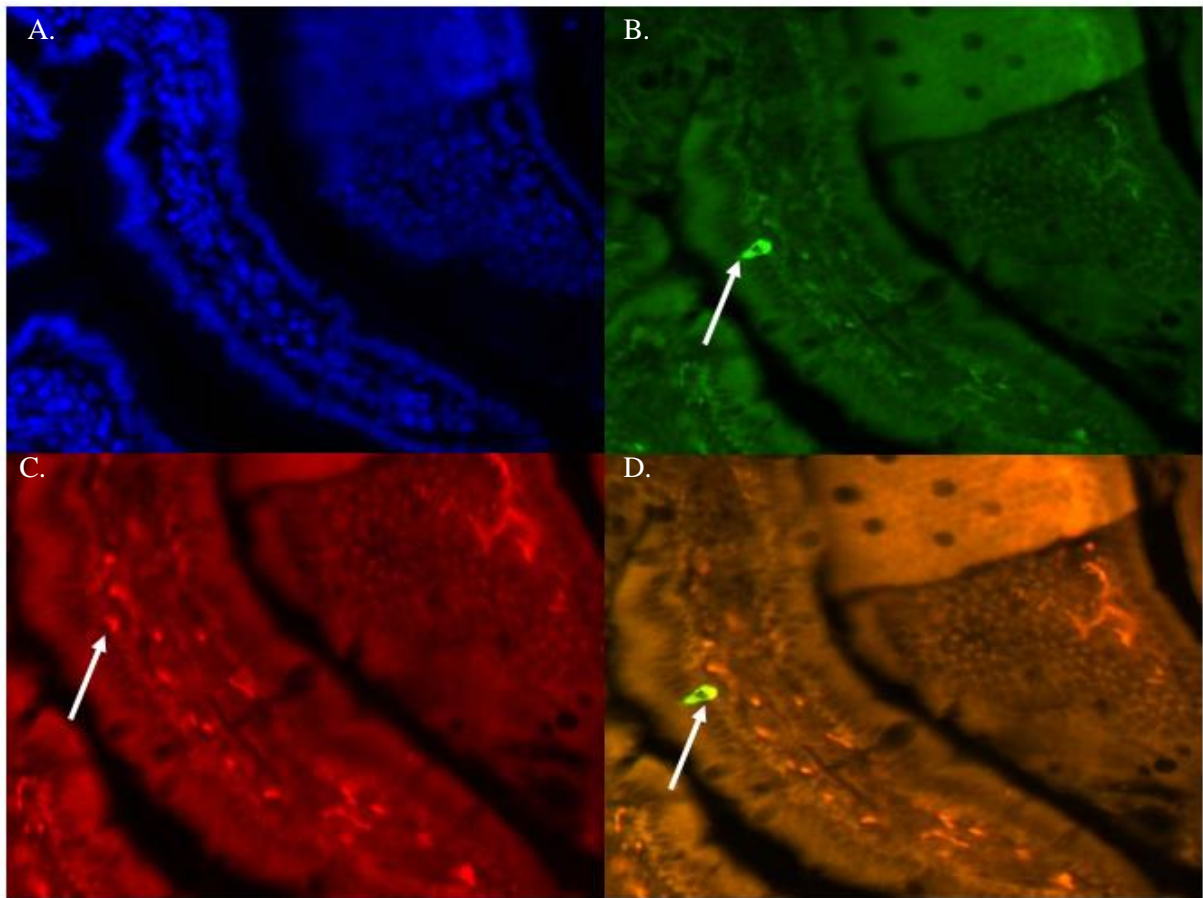
Distribution of (A) DAPI, (B) GIP, (C) GPR119 and (D) combined fluorescence of GIP and GPR119 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

Figure 7.21: Immunofluorescence staining of PYY and GPR119 in STC-1 cells



Distribution of (A) DAPI, (B) PYY, (C) GPR119 and (D) combined fluorescence of PYY and GPR119 in STC-1 cells. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

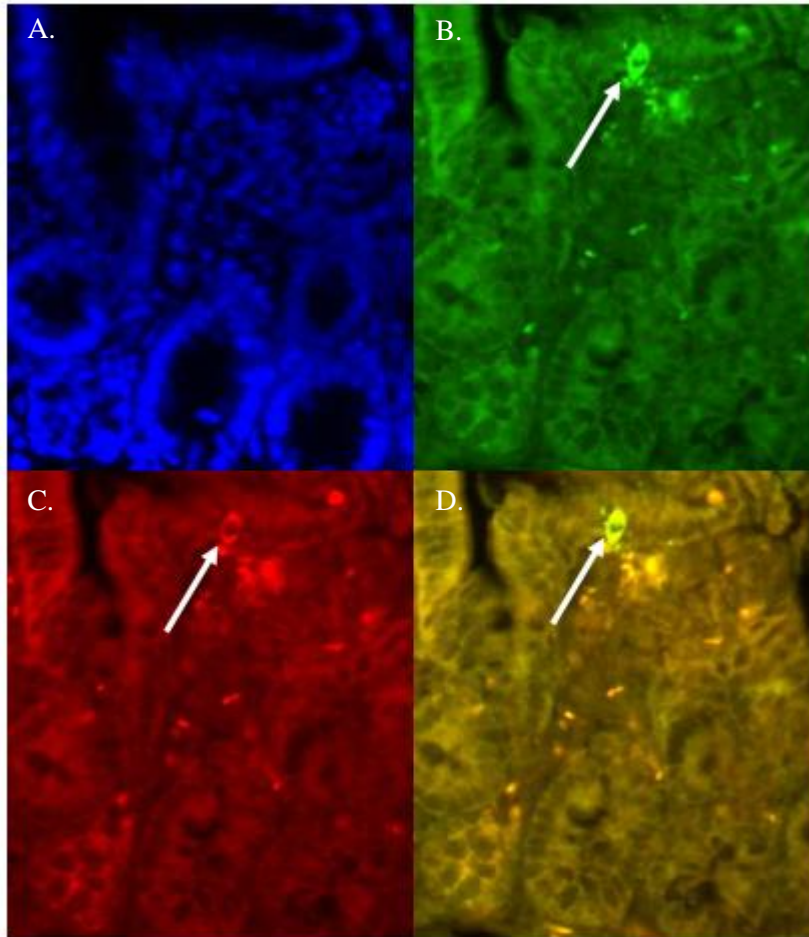
Figure 7.22: Immunofluorescence staining of PYY and GPR119 in lean NIH Swiss mouse small intestine



Distribution of (A) DAPI, (B) PYY, (C) GPR119 and (D) combined fluorescence of PYY and GPR119 in lean NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

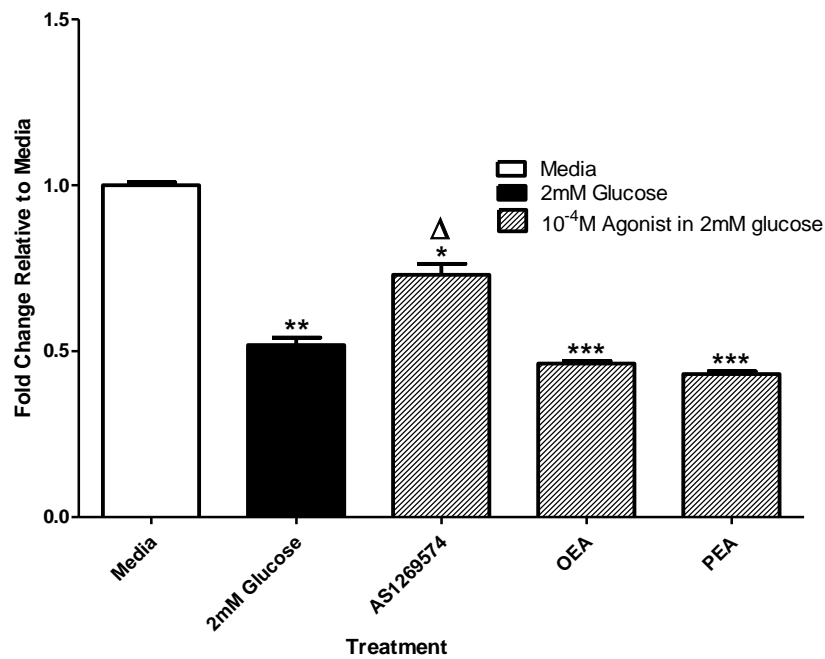
Figure 7.23: Immunofluorescence staining of PYY and GPR119 in HFF NIH Swiss mouse small intestine

B.



Distribution of (A) DAPI, (B) PYY, (C) GPR119 and (D) combined fluorescence of PYY and GPR119 in HFF NIH Swiss mouse small intestinal tissue. Images are taken at x200 magnification and examples of positive staining are indicated with white arrows.

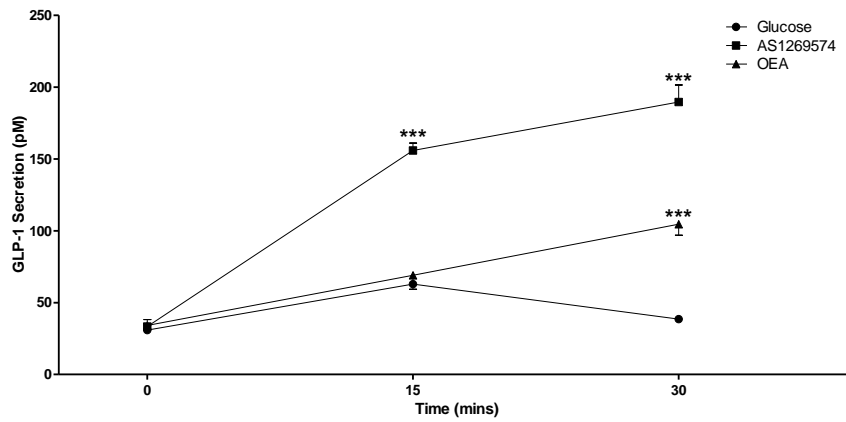
Figure 7.24: Effect of GPR119 agonists on GPR119 gene expression in GLUTag cells



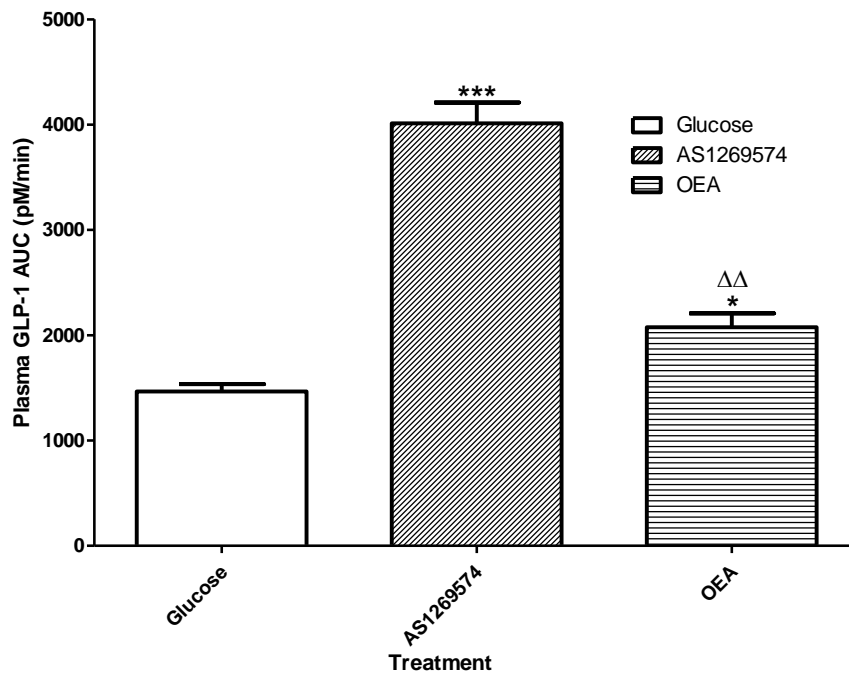
Effects of GPR119 agonists on the expression of the GPR119 gene in intestinal GLUTag cells at 2mM glucose (n=3). ** $p < 0.01$ and ** $p < 0.01$ compared to untreated media control. Δ $p < 0.05$ and ΔΔ $p < 0.01$ compared to vehicle glucose control.

Figure 7.25: Effects of GPR119 agonists on plasma GLP-1 in male Swiss TO mice

A.



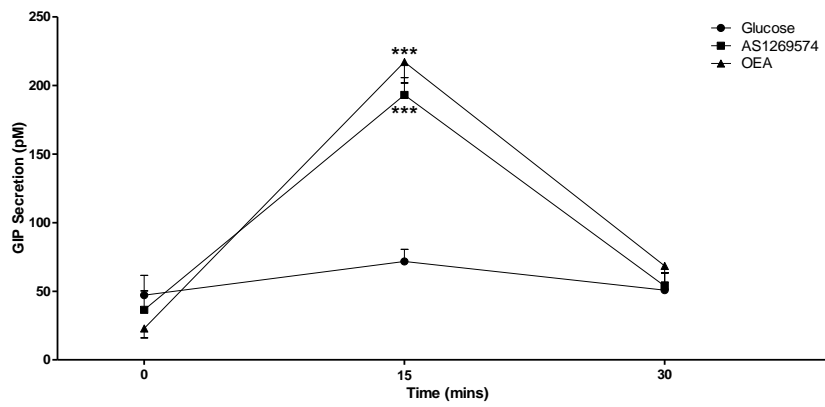
B.



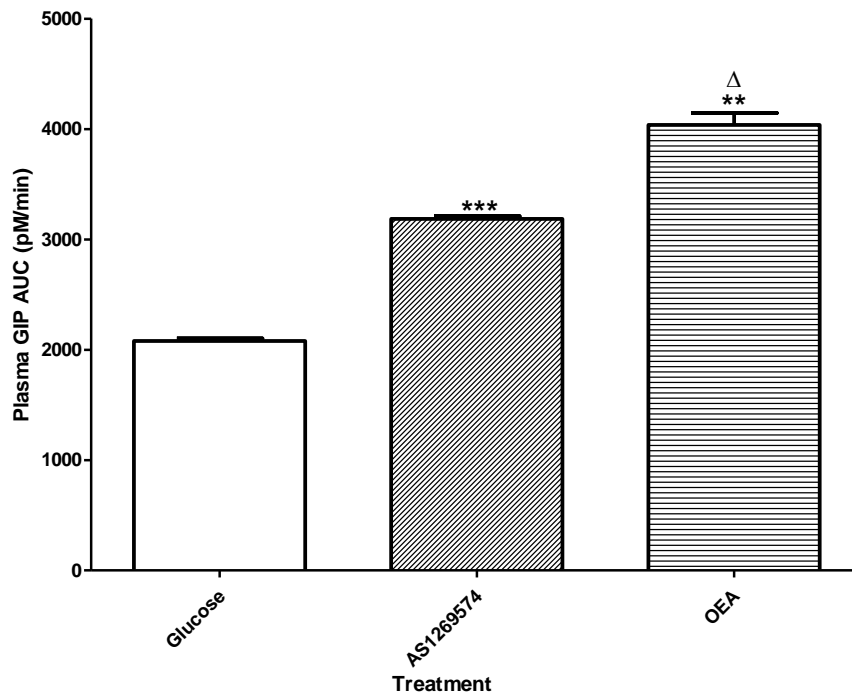
Glucose (18mmol/kg BW) or glucose in combination with a GPR119 agonist (0.1μmol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GLP-1 of mice treated with GPR119 agonists (B) AUC of A. * $p < 0.05$ and *** $p < 0.001$ compared to glucose treatment alone. ΔΔ $p < 0.01$ compared to AS1269574.

Figure 7.26: Effects of GPR119 agonists on plasma GIP in male Swiss TO mice

A.



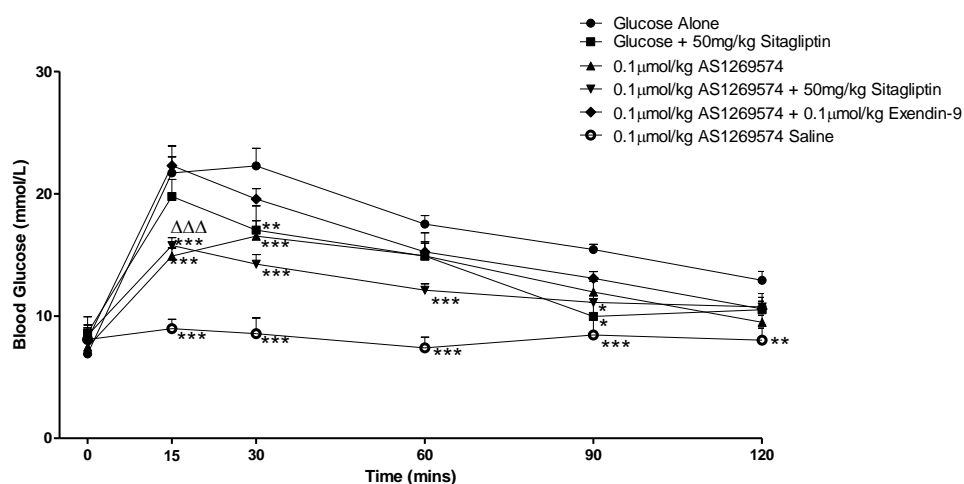
B.



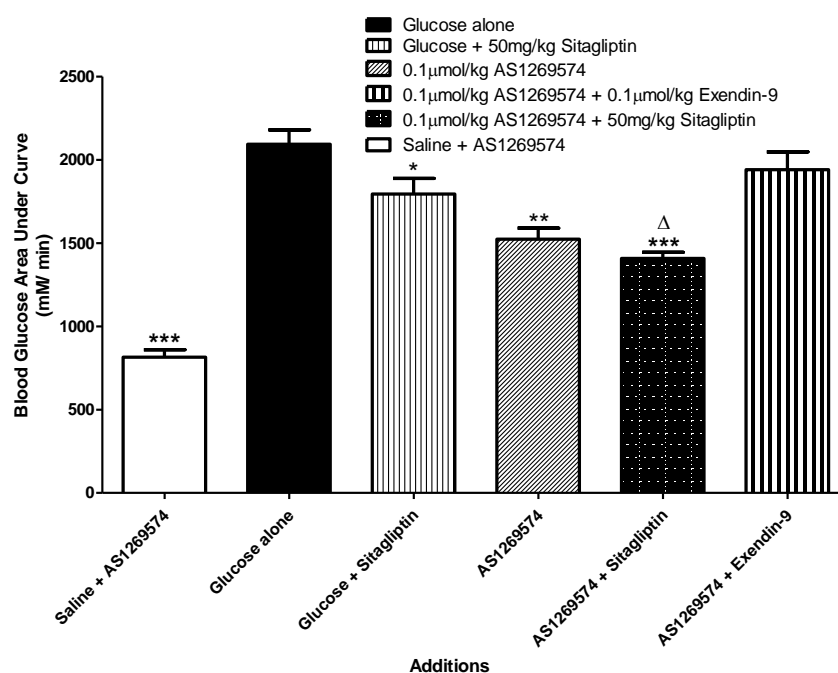
Glucose (18mmol/kg BW) or glucose in combination with a GPR119 agonist (0.1 μ mol/kg BW) were administered orally to male Swiss TO mice (n=6). (A) Plasma GIP of mice treated with GPR119 agonists (B) AUC of A. **p<0.01 and ***p<0.001 compared to glucose treatment alone. Δ p<0.05 compared to AS1269574.

Figure 7.27: Effects of AS1269574 on blood glucose in male Swiss TO mice

A.



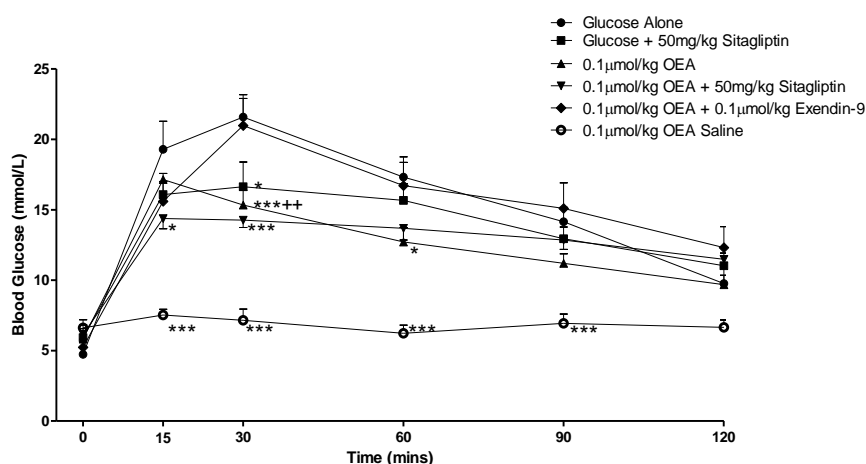
B.



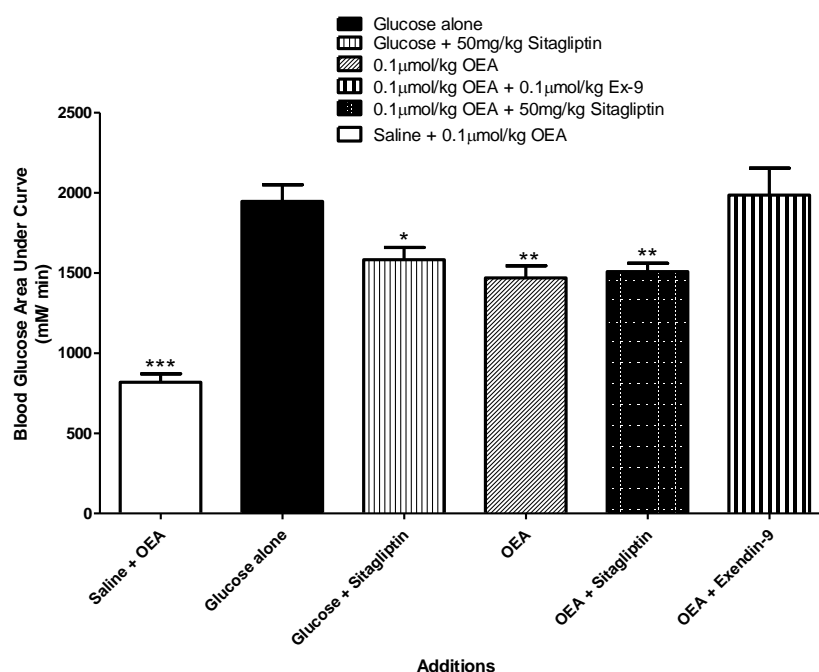
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with AS1269574 (0.1 μmol/kg BW), glucose in combination with AS1269574 and the GPR119 antagonist Exendin-9 (0.1 μmol/kg BW), glucose in combination with AS1269574 and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with AS1269574 were all administered orally to male Swiss TO mice. (A) Blood glucose of mice treated with AS1269574 (B) AUC of A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose alone. Δ $p < 0.05$ and ΔΔΔ $p < 0.001$ compared to glucose and Sitagliptin.

Figure 7.28: Effects of OEA on blood glucose in male Swiss TO mice

A.



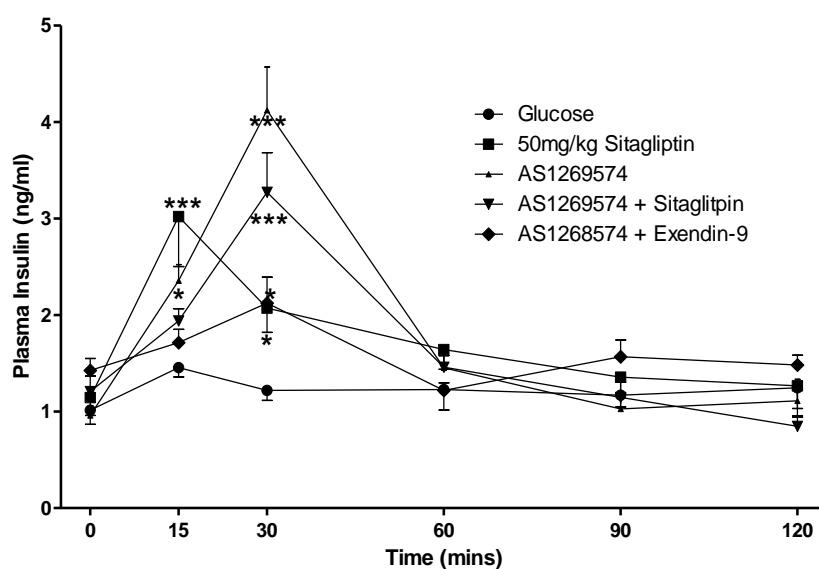
B.



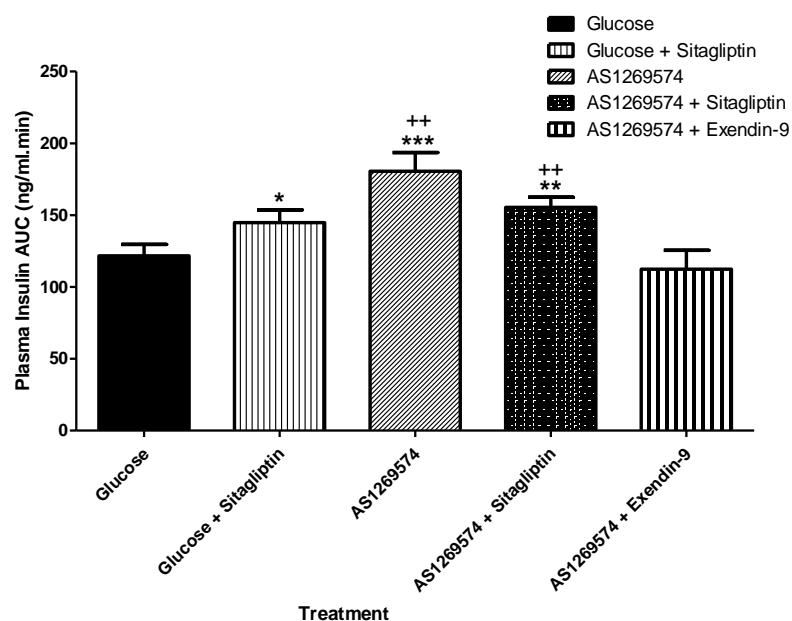
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with OEA (0.1µmol/kg BW), glucose in combination with OEA and the GPR119 antagonist Exendin-9 (0.1µmol/kg BW), glucose in combination with OEA and Sitagliptin Phosphate or saline (0.9% NaCl (w/v)) in combination with OEA were all administered orally to male Swiss TO mice. (A) Blood glucose of mice treated with OEA (B) AUC of A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose alone. ++ $p < 0.01$ compared to GPR119 agonist and Exendin-9.

Figure 7.29: Effects of AS1269574 on plasma insulin in male Swiss TO mice

A.



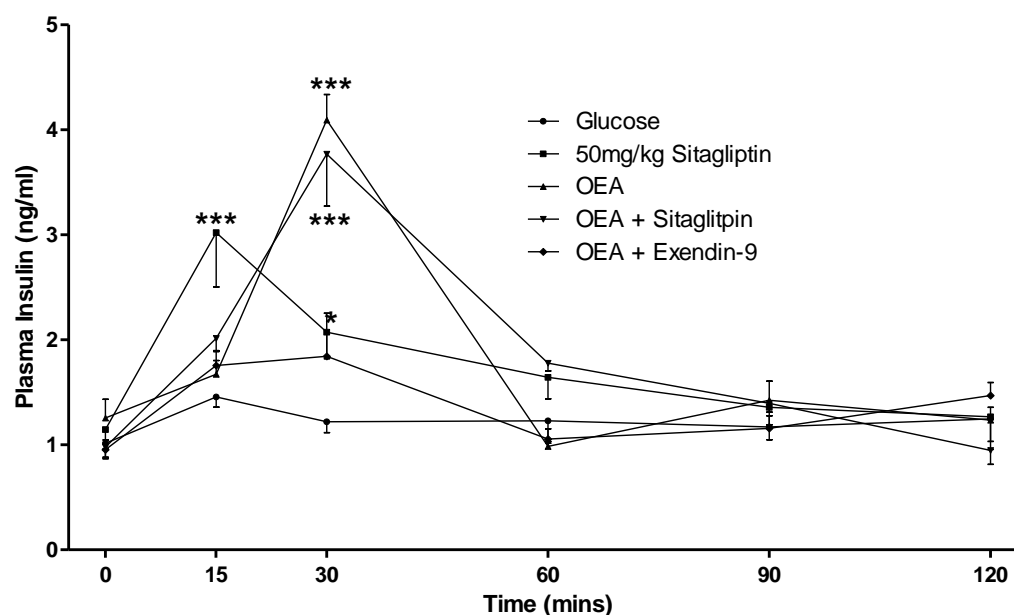
B.



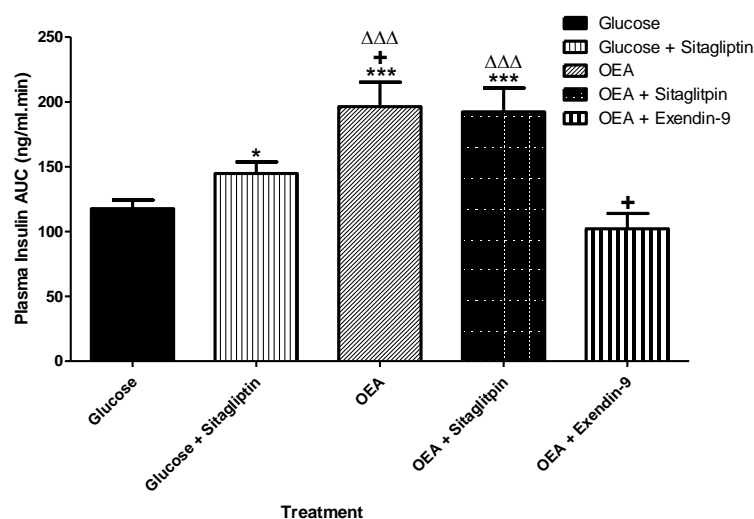
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with AS1269574 (0.1 μ mol/kg BW), and glucose in combination with AS1269574 and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with AS1269574 (B) AUC of A. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to glucose alone. ++ p < 0.01 compared to GPR119 agonist and Exendin-9.

Figure 7.30: Effects of OEA on plasma insulin in male Swiss TO mice

A.



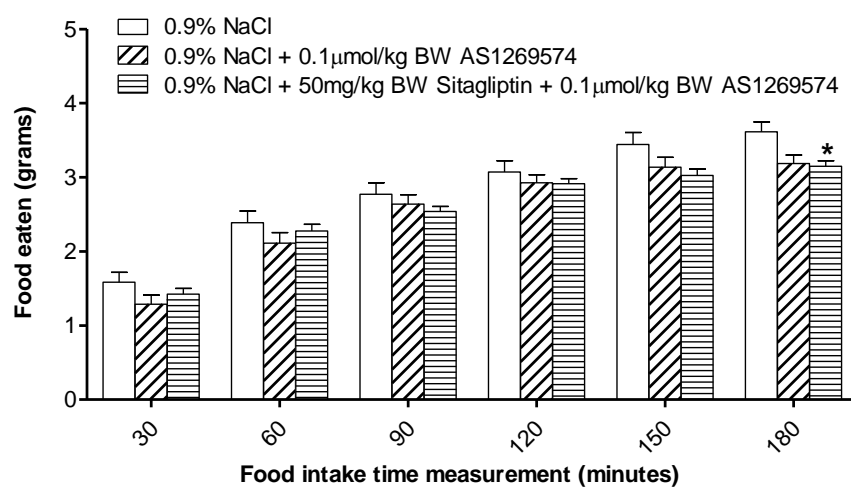
B.



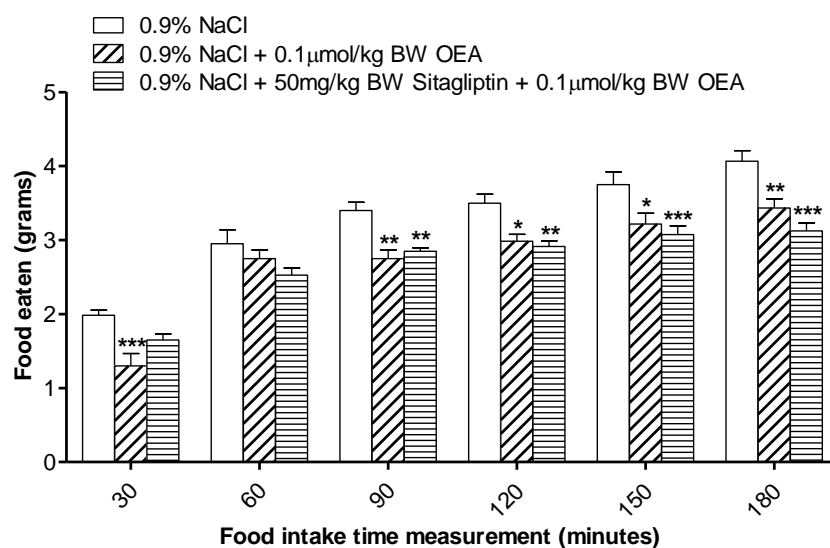
Glucose (18mmol/kg BW), glucose in combination with Sitagliptin Phosphate (50mg/kg BW), glucose in combination with OEA (0.1 μ mol/kg BW), and glucose in combination with OEA and Sitagliptin Phosphate were all administered orally to male Swiss TO mice (n=6). (A) Plasma insulin of mice treated with OEA (B) AUC of A (C) Plasma insulin of mice treated with OEA (D) AUC of C. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose alone. $\Delta\Delta\Delta$ $p < 0.001$ compared to GPR119 agonist and Exendin-9. + $p < 0.05$ compared to glucose and Sitagliptin.

Figure 7.31: Effects of GPR119 agonists on feeding in trained male Swiss TO mice

A.



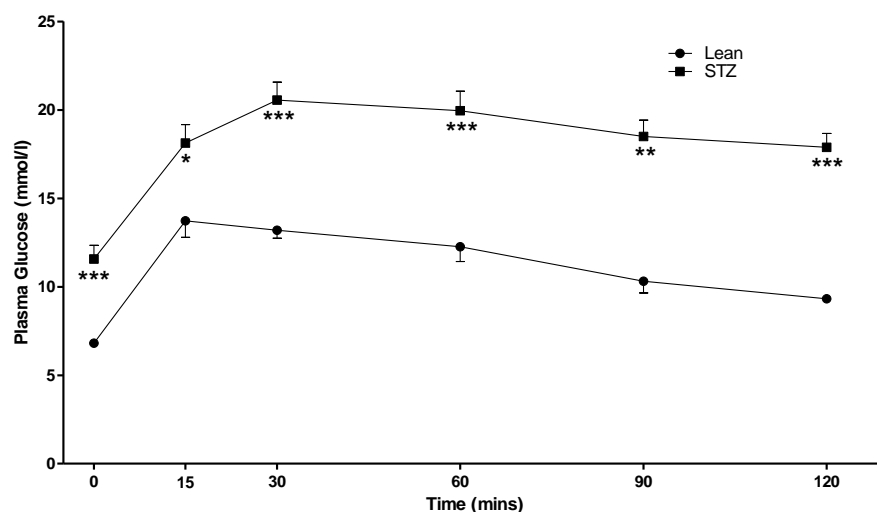
B.



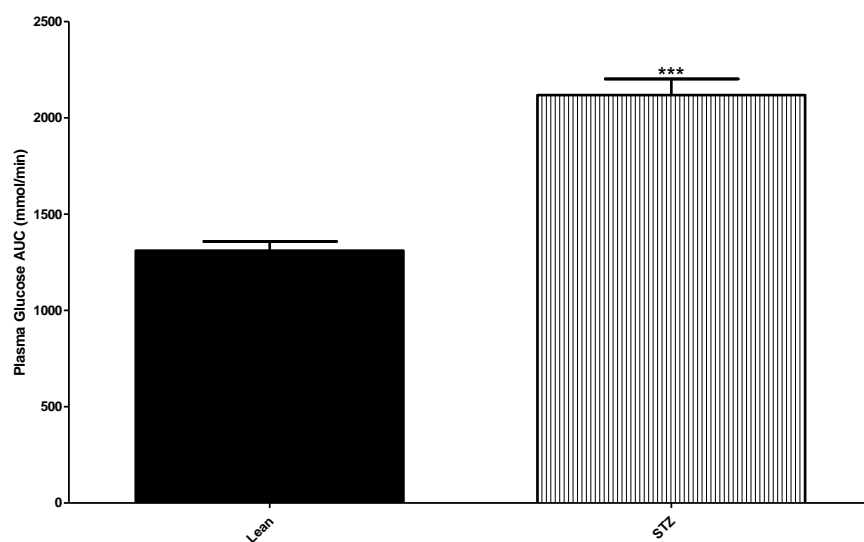
Saline (0.9%), saline and a GPR119 agonist (0.1 μmol/kg BW), saline with a GPR119 agonist and Sitagliptin (50mg/kg BW) or saline and Sitagliptin were administered orally to Swiss TO mice which had been trained to eat for 3 hrs daily (n=8). * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to saline. (A) Mice treated with AS1269574, (B) Mice treated with OEA.

Figure 7.32: Effect of multiple low dose streptozotocin on blood glucose and insulin in male Swiss TO mice

A.

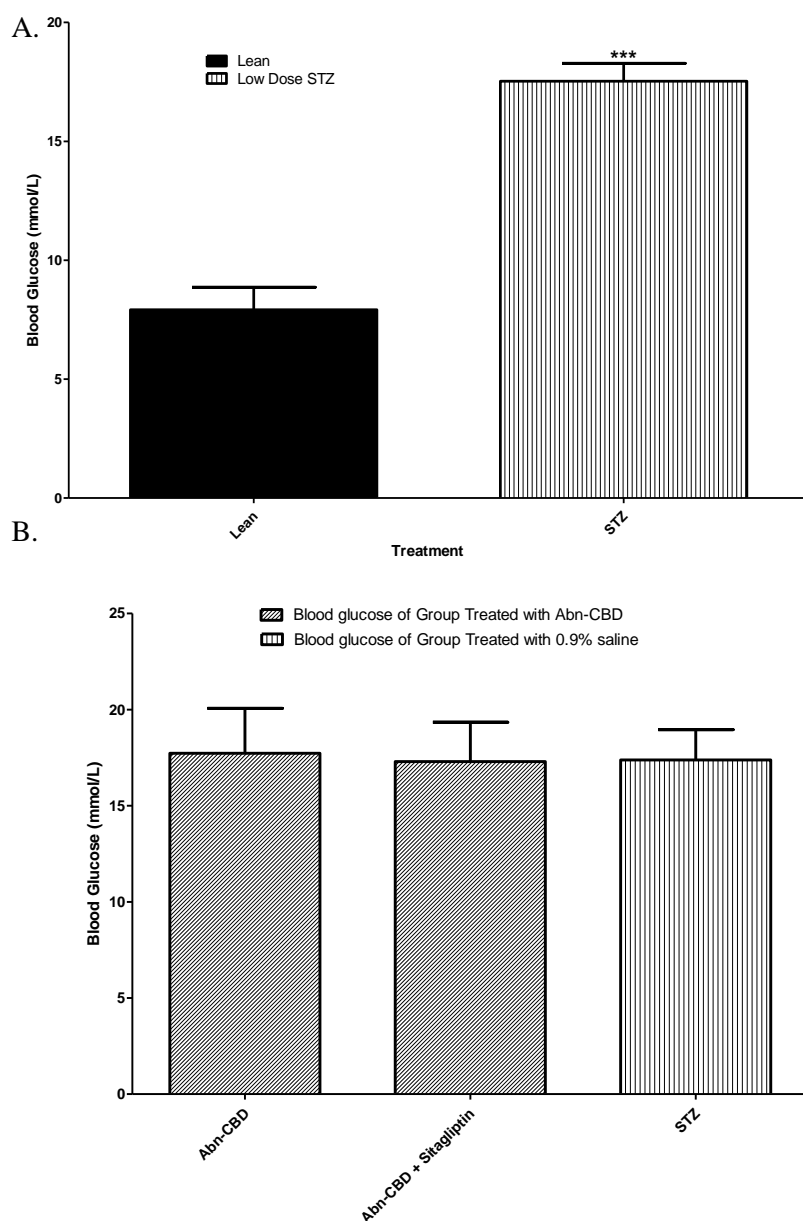


B.



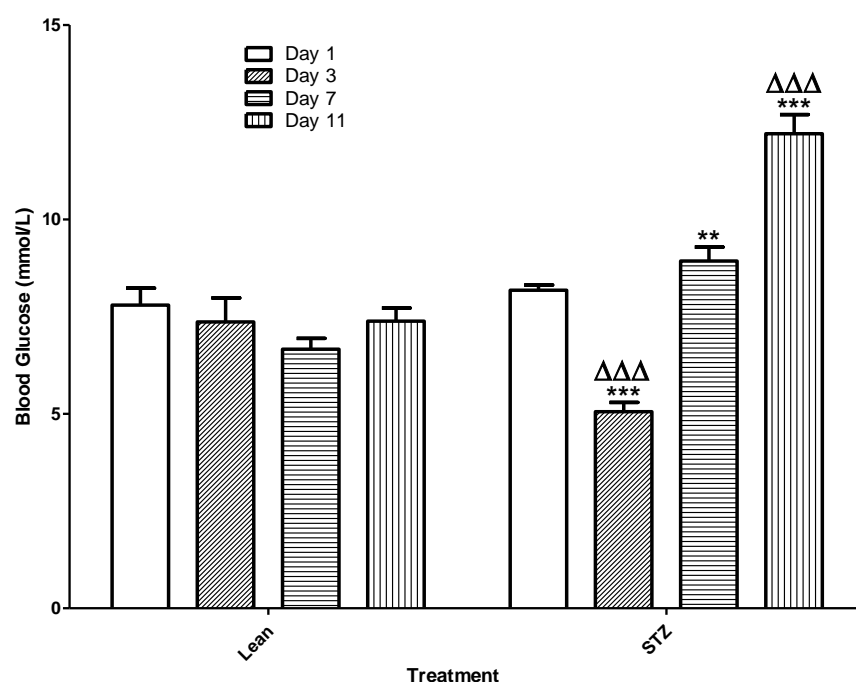
In order to induce diabetes, 4 hr fasted male Swiss TO mice were administered streptozotocin (40mg/kg BW, 0.1M sodium citrate, pH 4.5) via intraperitoneal injection. Reduced glucose tolerance was confirmed 14 days after the initial injection of streptozotocin (day 1) via oral glucose tolerance test. Glucose (18mmol/kg body weight) was administered orally to either lean or streptozotocin treated Swiss TO mice (n=6 for lean mice, n=12 for streptozotocin treated mice). (A) Blood glucose (B) AUC of A.

Figure 7.33: Effect of multiple low dose streptozotocin on non-fasting blood glucose in male Swiss TO mice



In order to induce diabetes, 4 hr fasted male Swiss TO mice were administered streptozotocin (40mg/kg BW, 0.1M sodium citrate, pH 4.5) via intraperitoneal injection. Non-fasting blood glucose was measured 14 days after initial injection (n=6 for lean and n=12 for streptozotocin induced diabetic mice) (A) Non fasting blood glucose of mice 14 days after initial treatment (B) Non-fasting blood glucose levels between treatment groups of streptozotocin treated mice when separated 14 days after initial treatment. Mice were separated to ensure no significant differences between groups before treatment commenced.

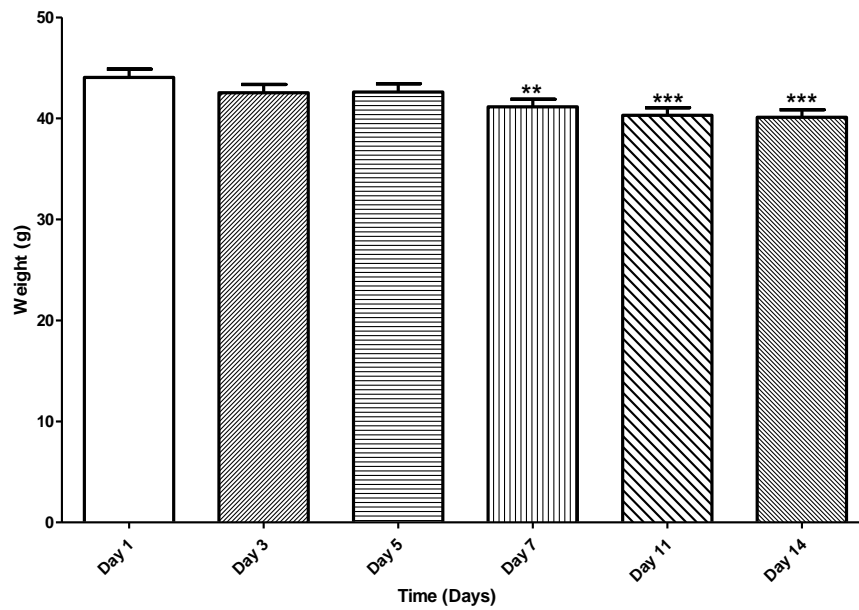
Figure 7.34: Effects of multiple low dose streptozotocin on blood glucose in 4 hr fasted male Swiss TO mice



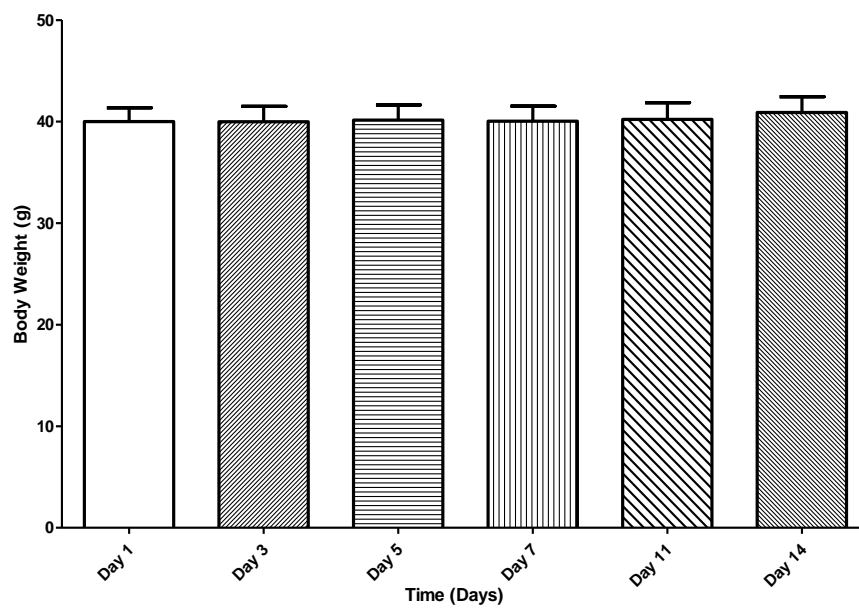
Male Swiss TO mice were fasted for 4 hrs prior to streptozotocin treatment to induce diabetes (n=32) or 0.9% saline (n=6) via intraperitoneal injection. Blood glucose was measured prior to injection. **p<0.01 and ***p<0.001 compared to lean blood glucose. ΔΔΔ p<0.001 compared to Day 1 blood glucose.

Figure 7.35: Effects of multiple low dose streptozotocin on body weight in male Swiss TO mice

A.



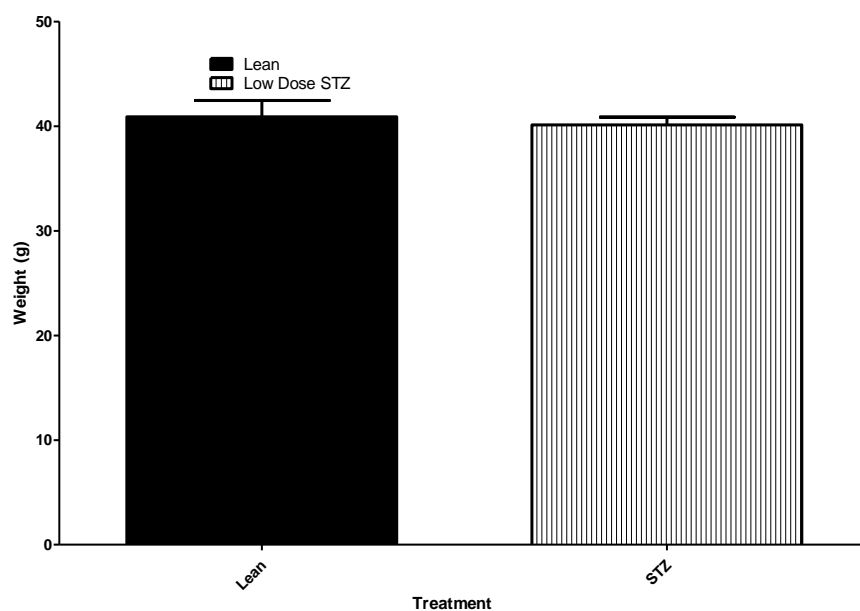
B.



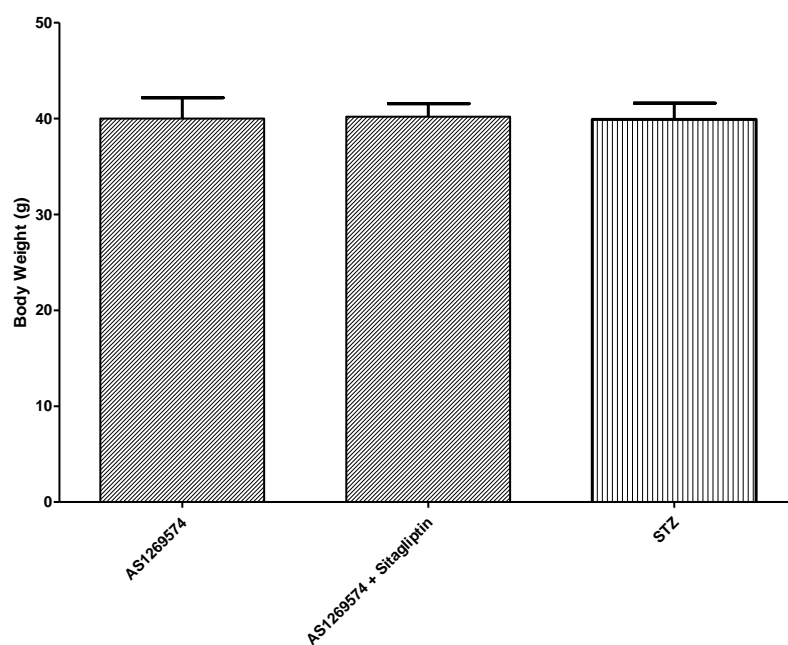
Body weights of both streptozotocin treated (A) and lean male Swiss TO mice (B) was monitored prior to injection (n=32 for streptozotocin treated mice, n=6 for lean mice). Lean mice received intraperitoneal injections of 0.9% saline in place of streptozotocin. ***p<0.001 compared to day 1.

Figure 7.36: Differences in body weight between treatment groups of streptozotocin induced diabetic male Swiss TO mice

A.



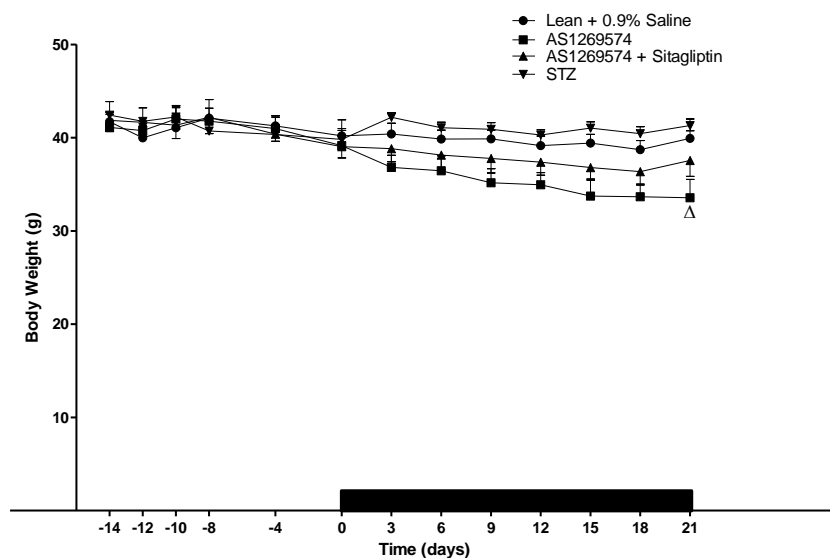
B.



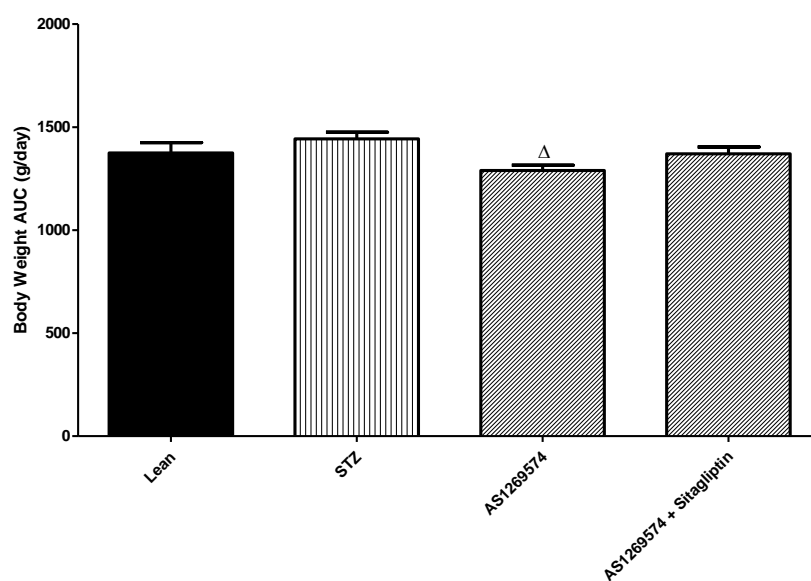
Body weight was compared between lean and streptozotocin treated mice 14 days after initial injection (A) and body weights were checked prior to initial agonist treatment (B).

Figure 7.37: Effects of long term daily oral treatment of GPR119 agonist AS1269574 body weight in multiple low dose streptozotocin mice

A.



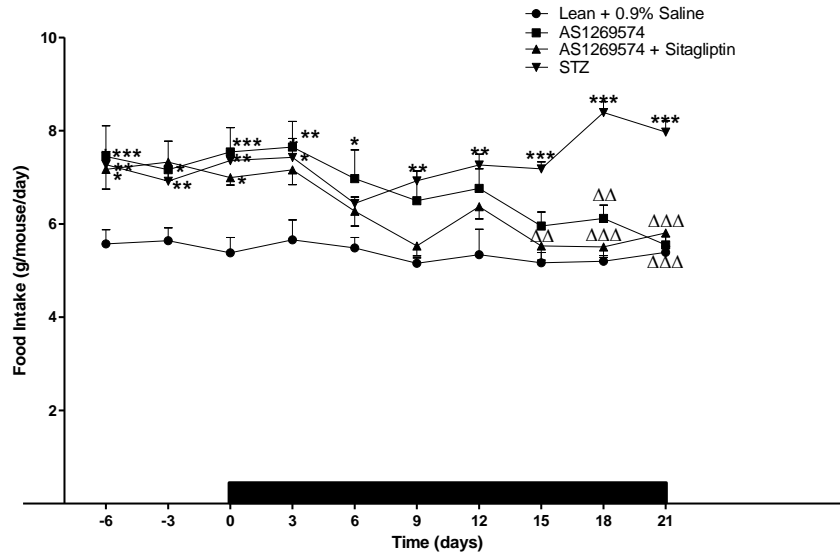
B.



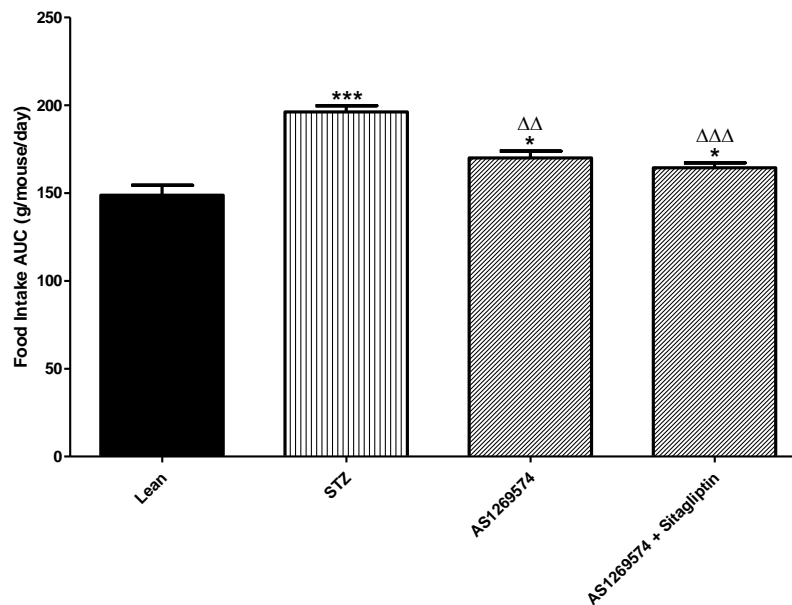
Effect of daily oral administration of saline (0.9%), AS1269574 monotherapy (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) on body weight. (A) Body weight (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). Δ p<0.05 compared to streptozotocin induced diabetic mice

Figure 7.38: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on food intake in multiple low dose streptozotocin mice

A.



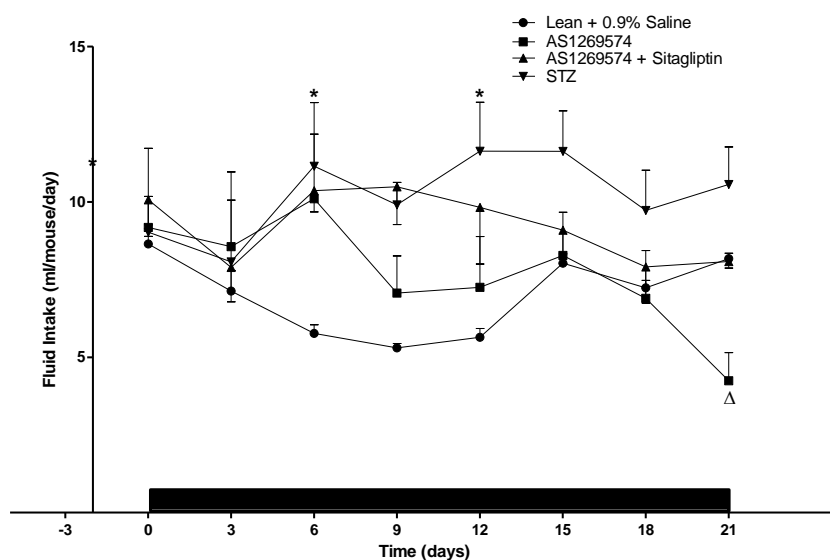
B.



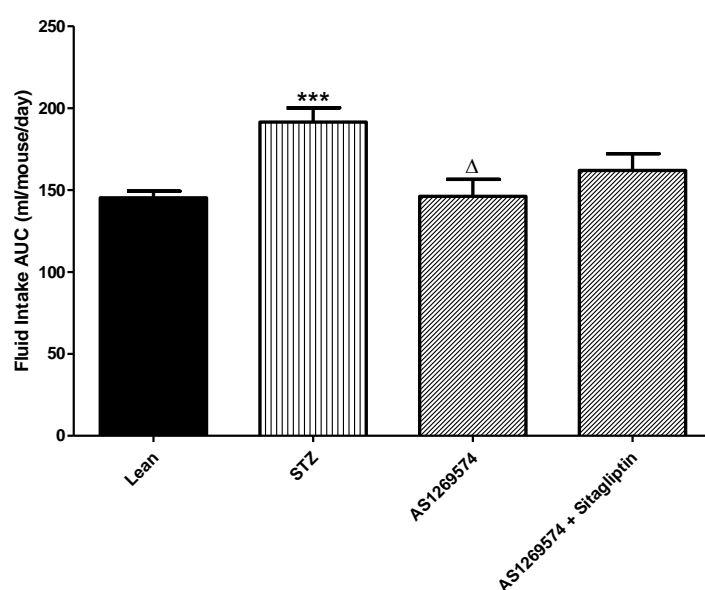
Effect of daily oral administration of saline (0.9%), AS1269574 monotherapy (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) on food intake. (A) Food intake (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin induced diabetic mice.

Figure 7.39: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on fluid intake in multiple low dose streptozotocin mice

A.



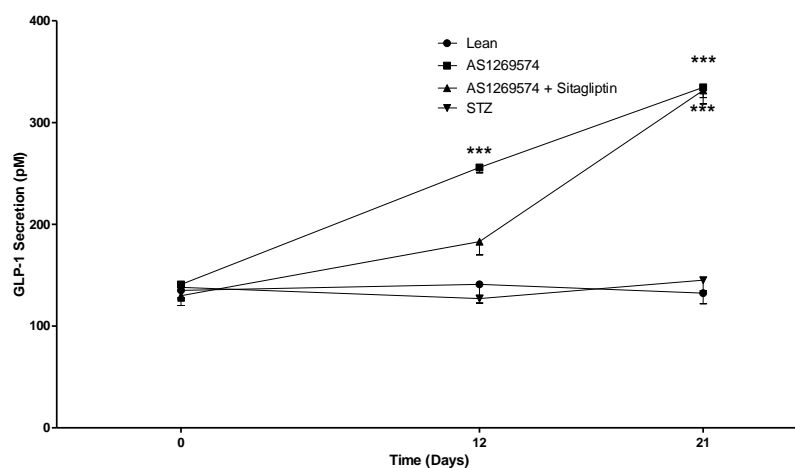
B.



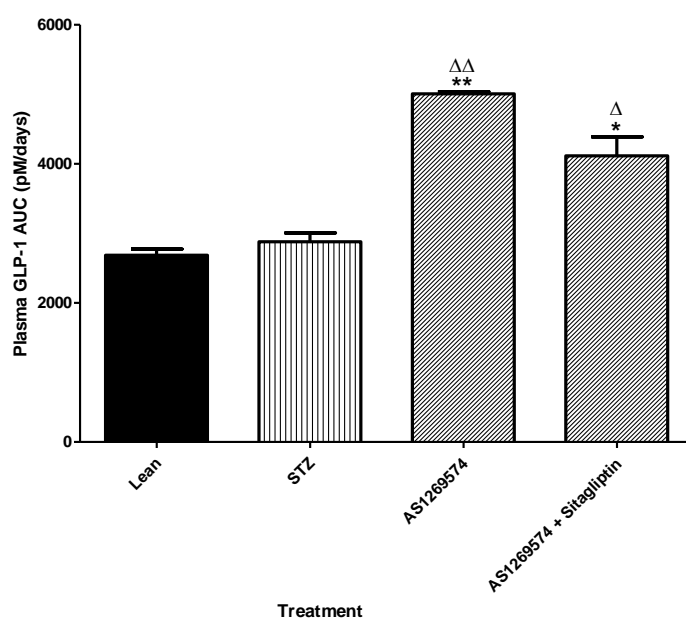
Effect of daily oral administration of saline (0.9%), AS1269574 monotherapy (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) combination therapy with Sitagliptin (50mg/kg body weight) on fluid intake. (A) Fluid intake (B) AUC of A. Parameters obtained before and during the 21 days treatment period (indicated by the horizontal black bar). Values are mean \pm SEM (n=6). * $p < 0.05$ and *** $p < 0.001$ compared to lean mice. Δ $p < 0.05$ compared to the streptozotocin group.

Figure 7.40: Effects of long term oral daily treatment of GPR119 agonist AS1269574 on plasma GLP-1 in multiple low dose streptozotocin mice

A.



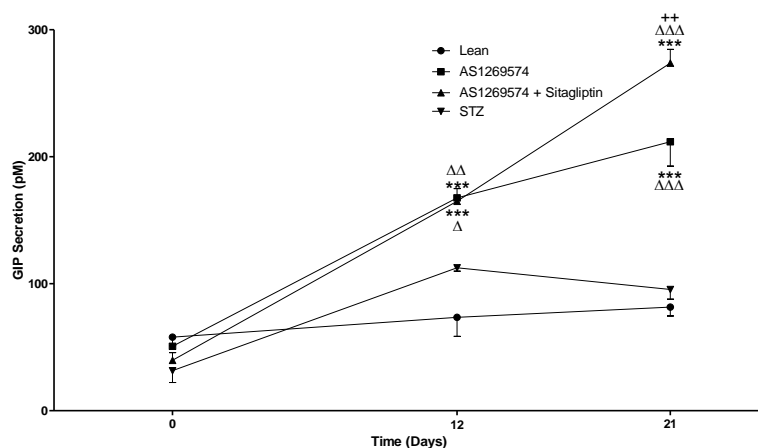
B.



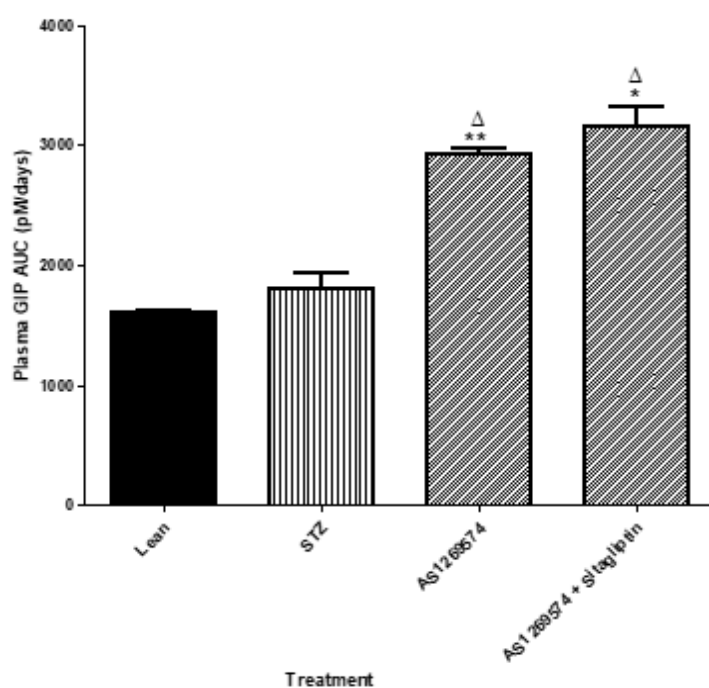
AS1269574 (0.1 μ mol/kg BW) in 0.9% saline or AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin Phosphate (50mg/kg body weight) were administered orally to male Swiss TO mice (n=6). Lean and streptozotocin induced diabetic mice were administered 0.9% saline orally (n=6). Effects of daily administration on (A) plasma GLP-1(B) AUC of plasma GLP-1. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. Δ $p < 0.05$ and $\Delta\Delta$ $p < 0.01$ compared to streptozotocin induced diabetic mice treated with 0.9% saline.

Figure 7.41: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on plasma GIP in multiple low dose streptozotocin mice

A.



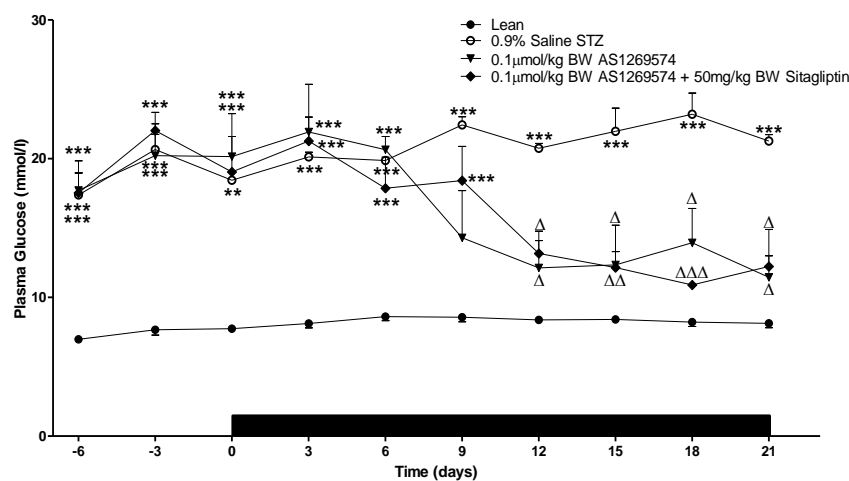
B.



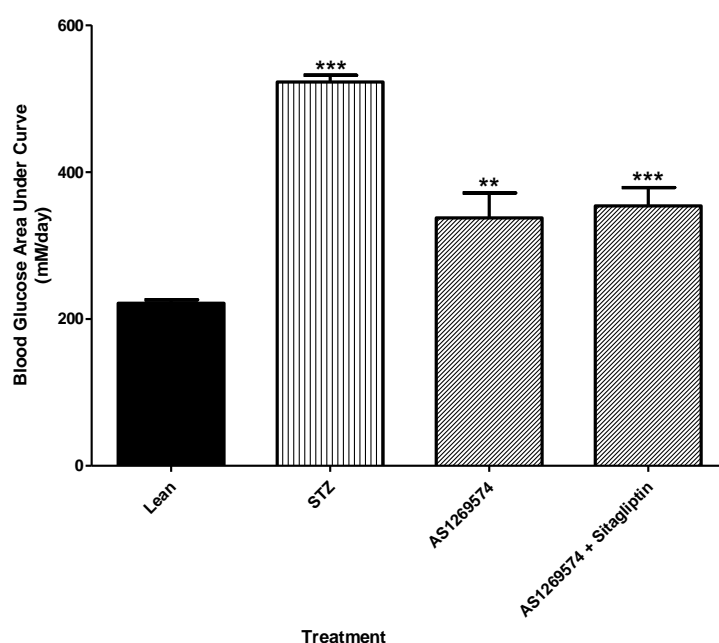
AS1269574 (0.1μmol/kg BW) in 0.9% saline or AS1269574 (0.1μmol/kg body weight) in combination with Sitagliptin Phosphate (50mg/kg body weight) were administered orally to male Swiss TO mice (n=6). Lean and streptozotocin induced diabetic mice were administered 0.9% saline orally (n=6). Effects of daily administration on (A) plasma GIP (B) AUC of plasma GIP. * p<0.05, **p<0.01 and ***p<0.001 compared to lean mice. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to streptozotocin induced diabetic mice treated with 0.9% saline.

Figure 7.42: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on blood glucose in multiple low dose streptozotocin mice

A.



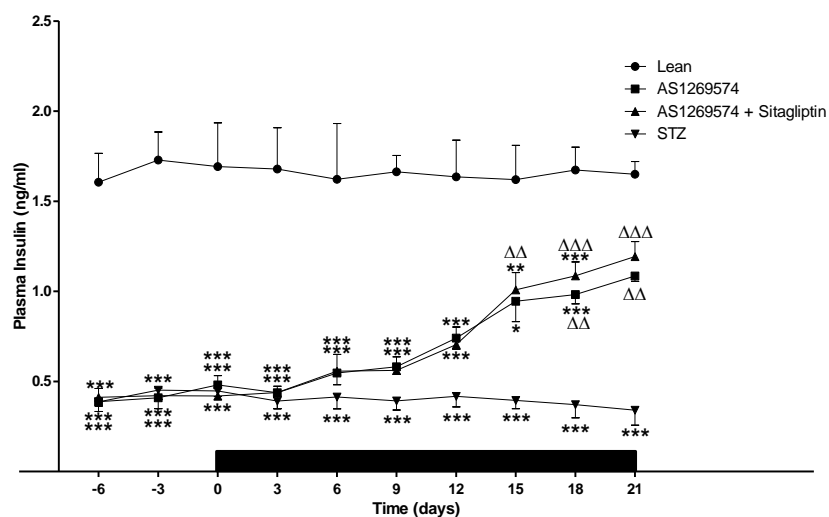
B.



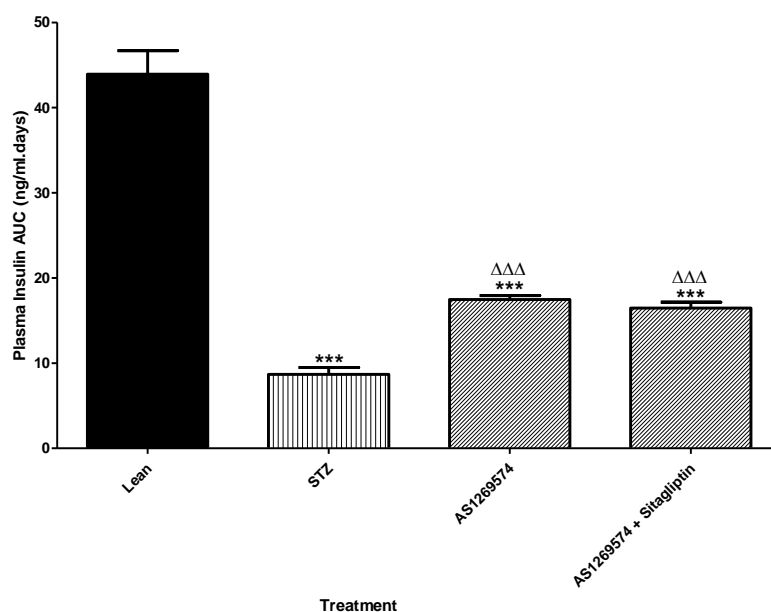
AS1269574 (0.1 μmol/kg BW) in 0.9% saline or AS1269574 in combination with Sitagliptin Phosphate were administered orally to male Swiss TO mice (n=6). Lean and streptozotocin induced diabetic mice were administered 0.9% saline orally (n=6). Effects of daily administration on (A) Blood glucose (B) AUC of A. **p<0.01 and ***p<0.001 compared to lean mice. Δ p<0.05, ΔΔ p<0.01 and ΔΔΔ p<0.001 compared to streptozotocin induced diabetic mice treated with 0.9% saline.

Figure 7.43: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on plasma insulin in multiple low dose streptozotocin mice

A.



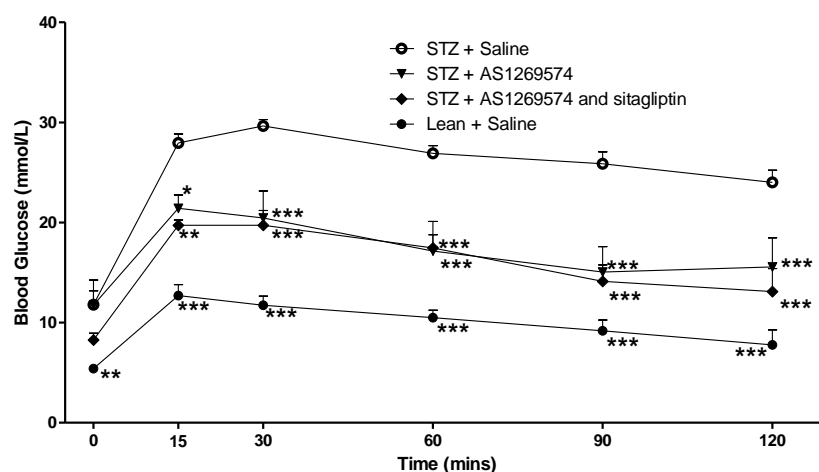
B.



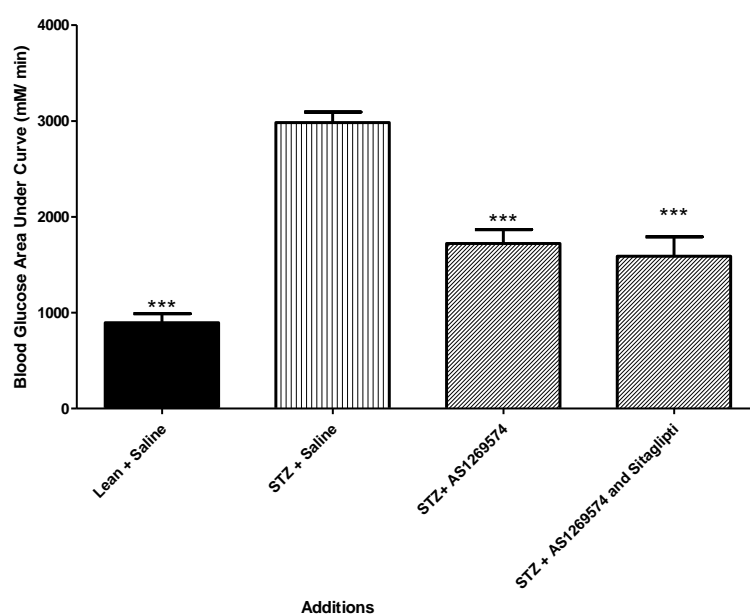
AS1269574 (0.1 μ mol/kg BW) in 0.9% saline or AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin Phosphate (50mg/kg body weight) were administered orally to male Swiss TO mice (n=6). Lean and streptozotocin induced diabetic mice were administered 0.9% saline orally (n=6). Effects of daily administration on (A) Plasma insulin (B) AUC of A. * p<0.05, **p<0.01 and ***p<0.001 compared to lean mice. Δ p<0.05, $\Delta\Delta$ p<0.01 and $\Delta\Delta\Delta$ p<0.001 compared to streptozotocin induced diabetic mice treated with 0.9% saline.

Figure 7.44: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on glucose tolerance in multiple low dose streptozotocin mice

A.



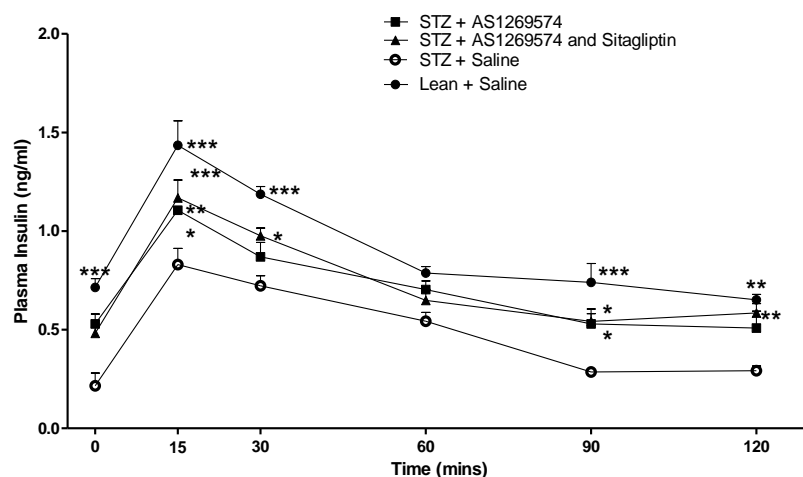
B.



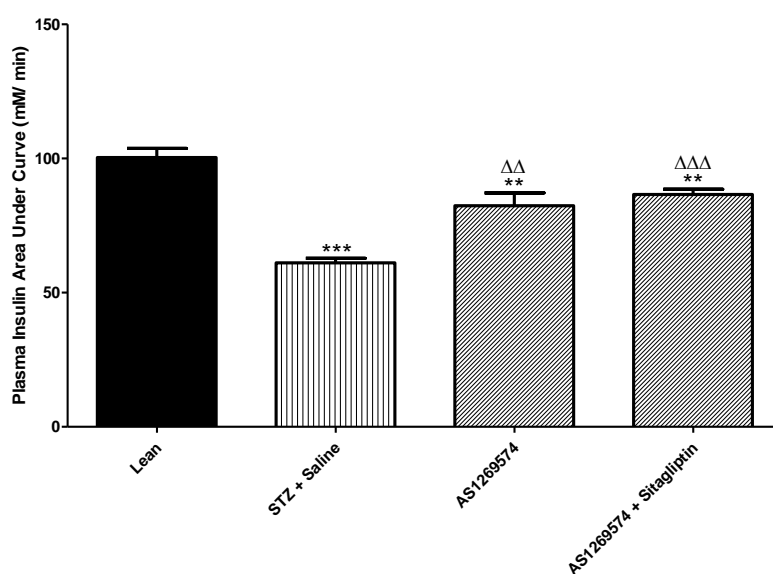
Mice treated with orally with GPR119 agonist AS1269574 (0.1 μ mol/kg body weight) or AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) and mice treated with saline (0.9%) underwent an oral glucose tolerance test (18mmol/kg body weight) 21 days after treatment. Mice were fasted for 18 hrs before glucose administration. (A) Blood glucose, (B) AUC of blood glucose. Results are the mean \pm SEM (n=6). * p<0.05, ** p<0.01 and *** p<0.001 compared to lean mice.

Figure 7.45: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on acute insulin secretion in multiple low dose streptozotocin mice

A.



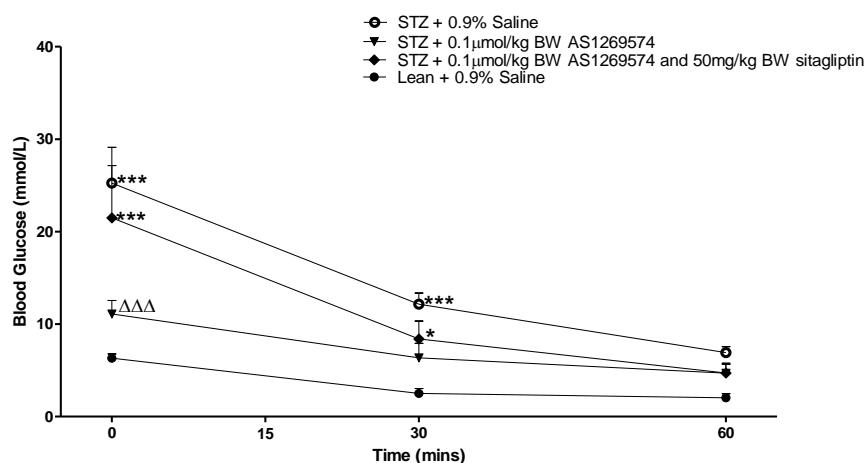
B.



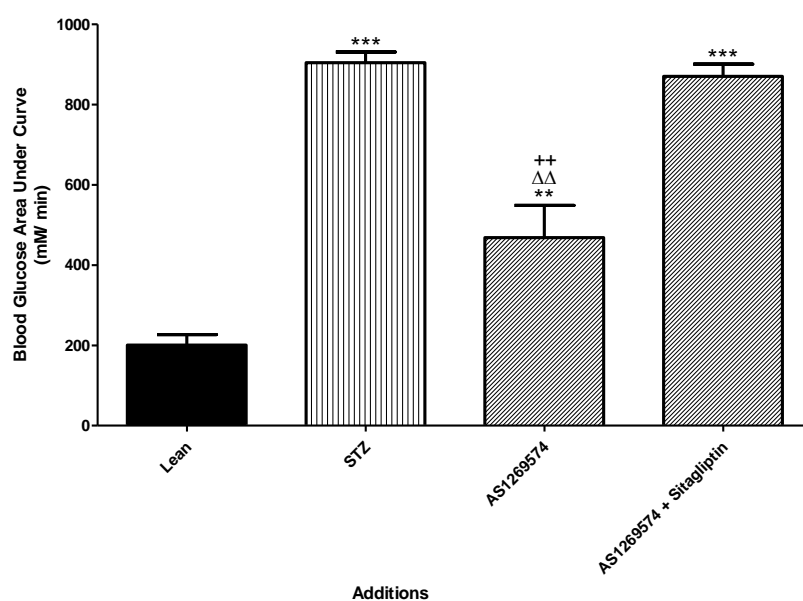
Mice treated with orally with GPR119 agonist AS1269574 (0.1 μ mol/kg body weight) or AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) and mice treated with saline (0.9%) underwent an oral glucose tolerance test (18mmol/kg body weight) 21 days after treatment. Mice were fasted for 18 hrs before glucose administration. (A) Plasma insulin, and (B) AUC of plasma insulin. Results are the mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin group.

Figure 7.46: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on insulin sensitivity in multiple low dose streptozotocin mice

A.



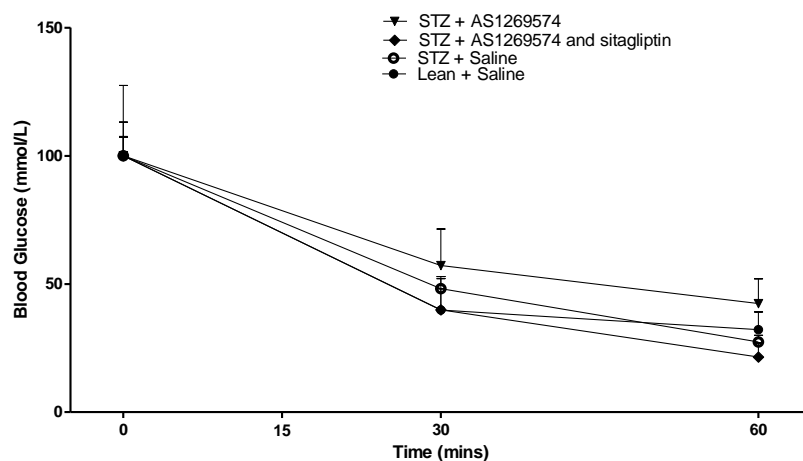
B.



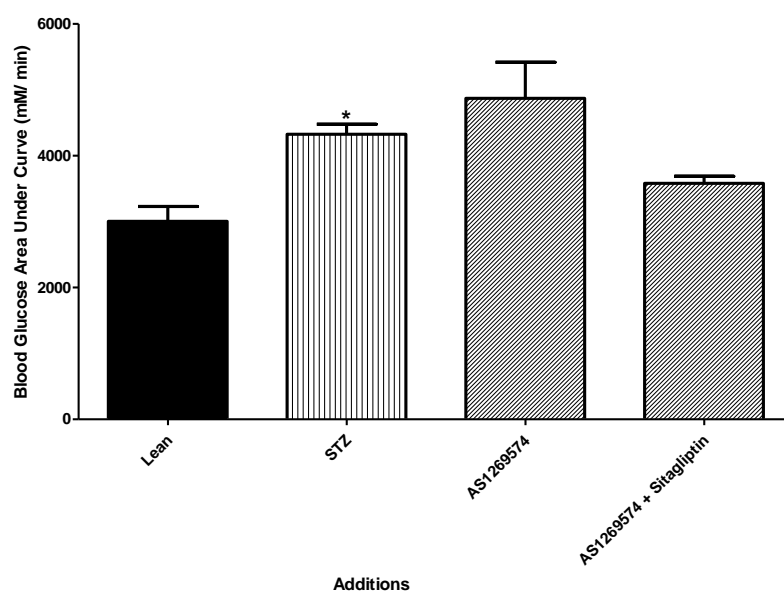
Non fasted Swiss TO mice were administered insulin via intraperitoneal injection (25 U/kg body weight, dissolved in 0.9% NaCl) 21 days after treatment with saline (0.9%), AS-1269574 (0.1 $\mu\text{mol/kg}$ body weight) or AS1269574 (0.1 $\mu\text{mol/kg}$ body weight) in combination with Sitagliptin (50mg/kg body weight). (A) Blood glucose, (B) AUC of blood glucose. Results are the mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to lean mice. $\Delta\Delta$ $p < 0.01$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to diabetic mice. ++ $p < 0.01$ compared to AS1269574 in combination with Sitagliptin.

Figure 7.47: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on insulin sensitivity in multiple low dose streptozotocin mice

A.



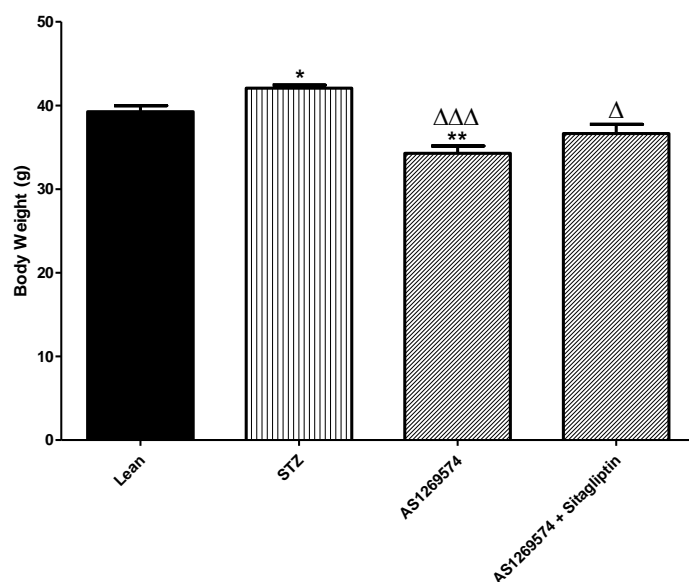
B.



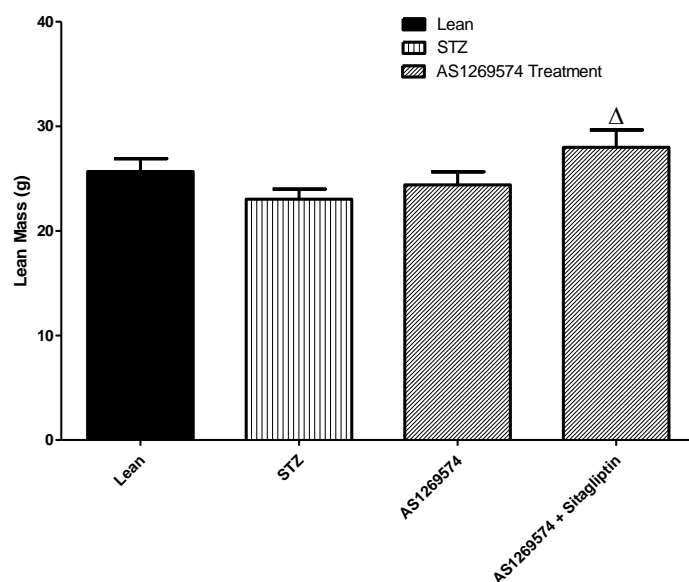
Non fasted Swiss TO mice were administered insulin via intraperitoneal injection (25 U/kg body weight, dissolved in 0.9% NaCl) 21 days after treatment with saline (0.9%), AS-1269574 (0.1 μ mol/kg body weight) or AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight). (A) Blood glucose (% change), and (B) AUC of blood glucose (% change) are shown. Results are the mean \pm SEM (n=6). * $p < 0.05$ compared to lean mice.

Figure 7.48: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on body weight and lean mass as measured by DEXA scanning in multiple low dose streptozotocin mice

A.



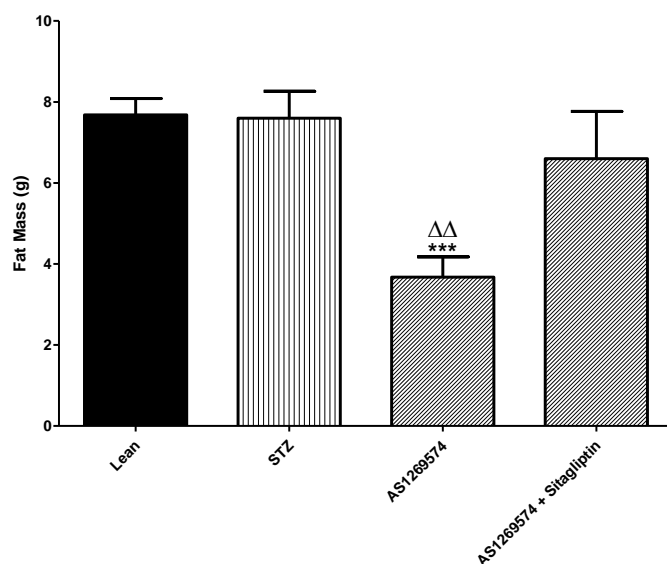
B.



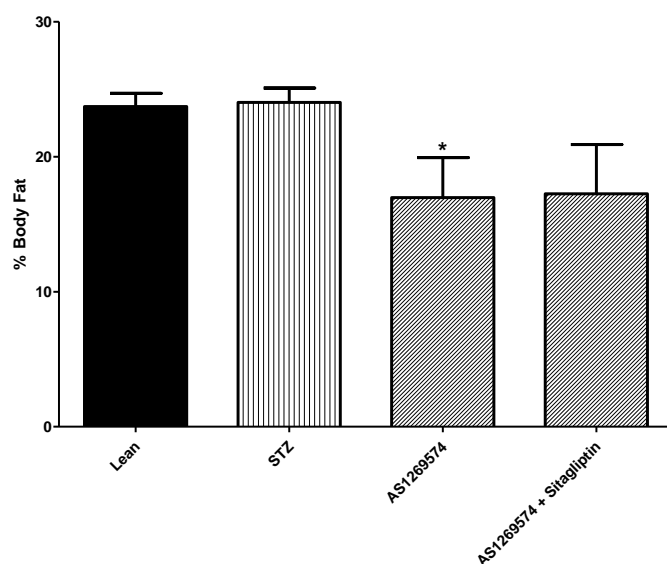
Effect of daily oral administration of saline (0.9%), AS-1269574 (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) in Swiss TO mice on (A) body weight and (B) lean mass as measured by DEXA scanning following 21 day treatment. Values are mean \pm SEM (n=6). * $p < 0.05$ and ** $p < 0.01$ compared lean mice. Δ $p < 0.05$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin induced diabetic mice.

Figure 7.49: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on fat mass and % body fat as measured by DEXA scanning in multiple low dose streptozotocin mice

A.

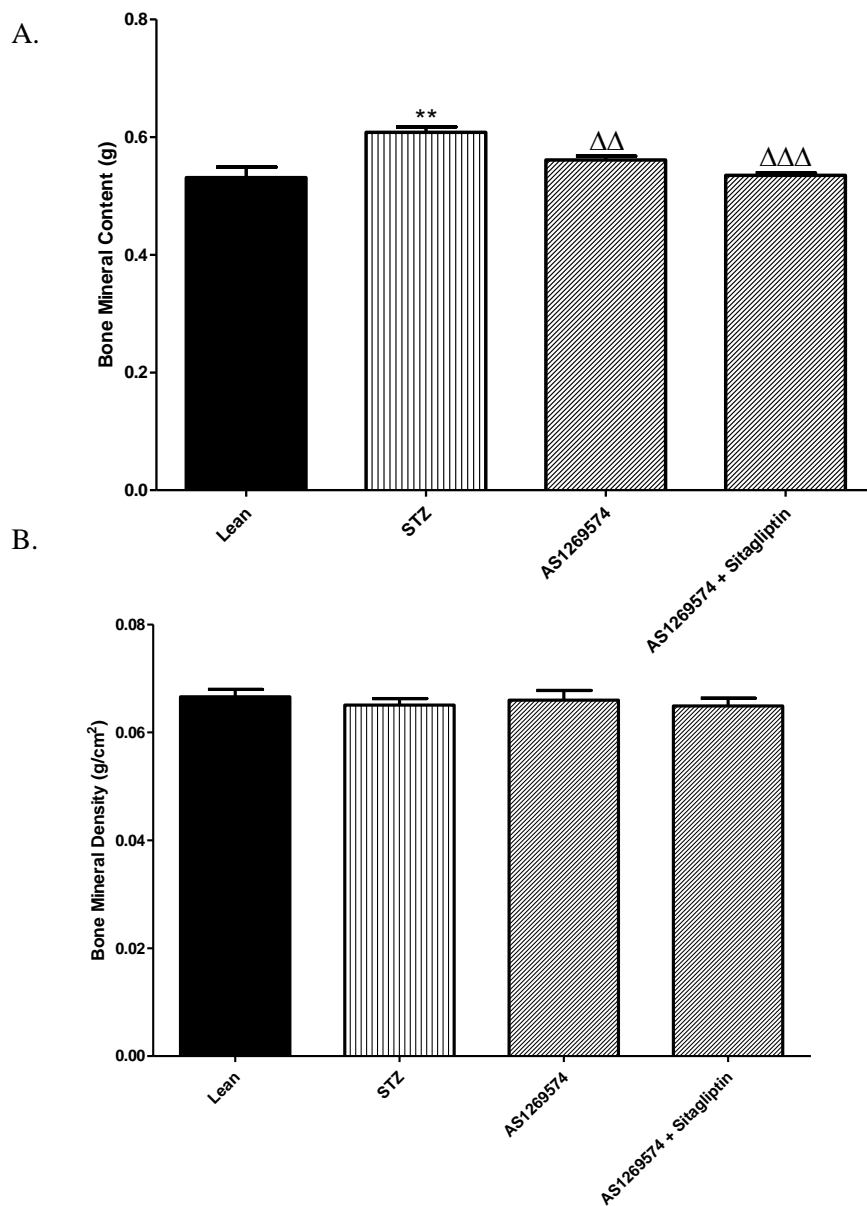


B.



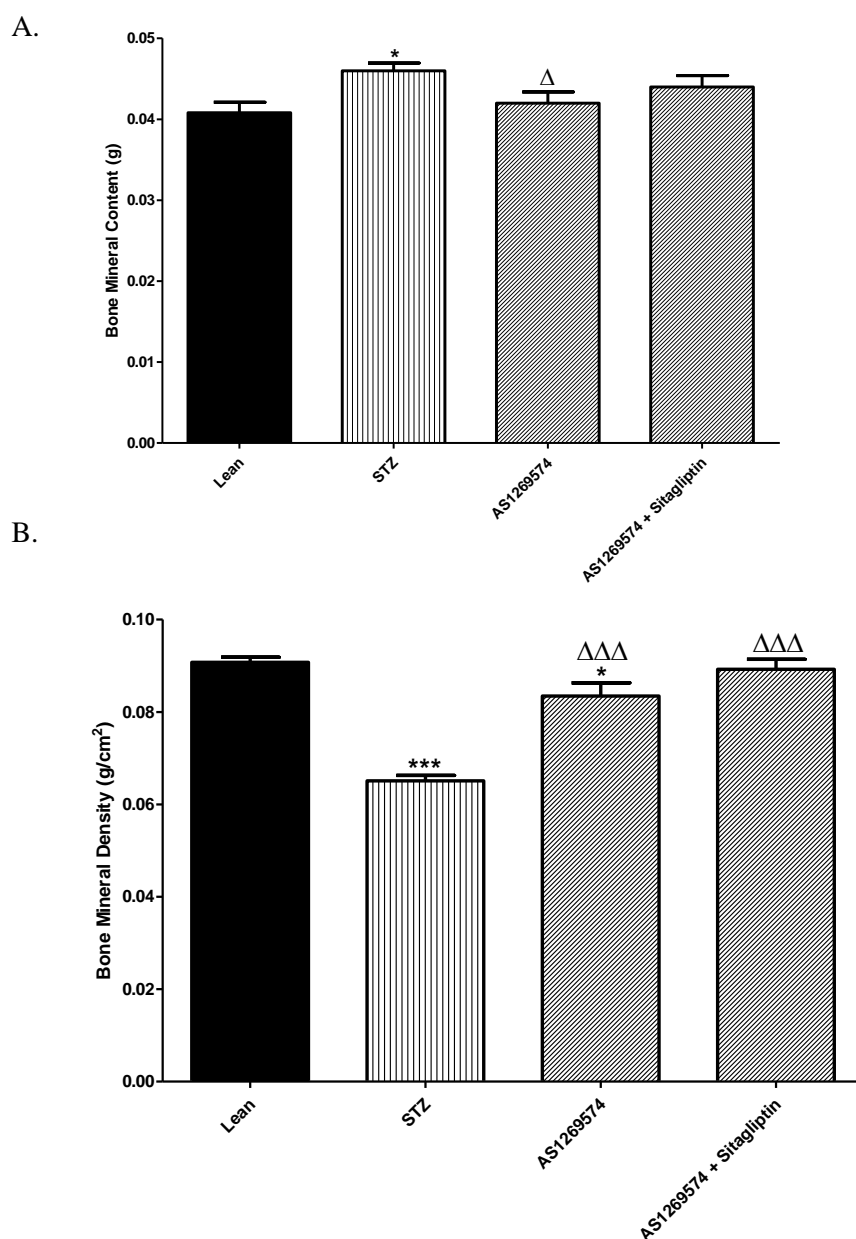
Effect of daily oral administration of saline (0.9%), AS-1269574 (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) in Swiss TO mice on (A) Fat mass and (B) % body fat as measured by DEXA scanning following 21 day treatment. Values are mean \pm SEM (n=6). * $p < 0.05$ and *** $p < 0.001$ compared lean mice. $\Delta\Delta$ $p < 0.01$ compared to streptozotocin induced diabetic mice.

Figure 7.50: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on bone mineral density and content as measured by DEXA scanning in multiple low dose streptozotocin mice



Effect of daily oral administration of saline (0.9%), AS-1269574 (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) in Swiss TO mice on (A) bone mineral content (B) bone mineral density as measured by DEXA scanning following 21 day treatment. Values are mean \pm SEM (n=6). ** p<0.01 compared lean mice. $\Delta\Delta$ p<0.01 and $\Delta\Delta\Delta$ p<0.001 compared to streptozotocin induced diabetic mice.

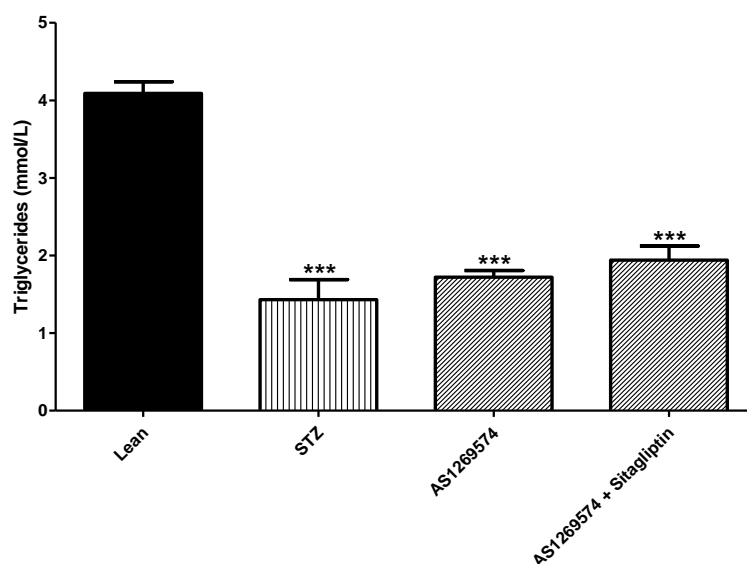
Figure 7.51: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on bone mineral density and content in the femur as measured by DEXA scanning in multiple low dose streptozotocin mice



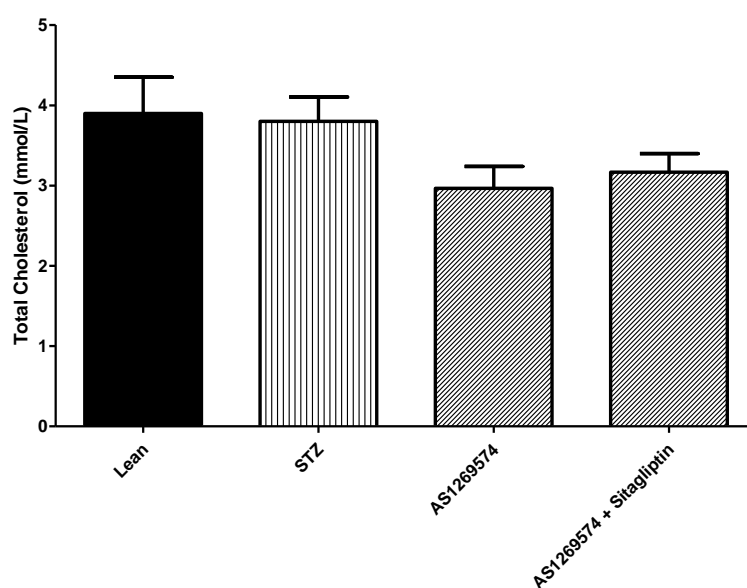
Effect of daily oral administration of saline (0.9%), AS-1269574 (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) in combination with Sitagliptin (50mg/kg body weight) in Swiss TO mice on (A) bone mineral content (B) bone mineral density in the femur as measured by DEXA scanning following 21 day treatment. Values are mean \pm SEM (n=6). * $p < 0.05$ and *** $p < 0.001$ compared lean mice. Δ $p < 0.05$ and $\Delta\Delta\Delta$ $p < 0.001$ compared to streptozotocin induced diabetic mice.

Figure 7.52: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on plasma triglycerides and total cholesterol in multiple low dose streptozotocin mice

A.



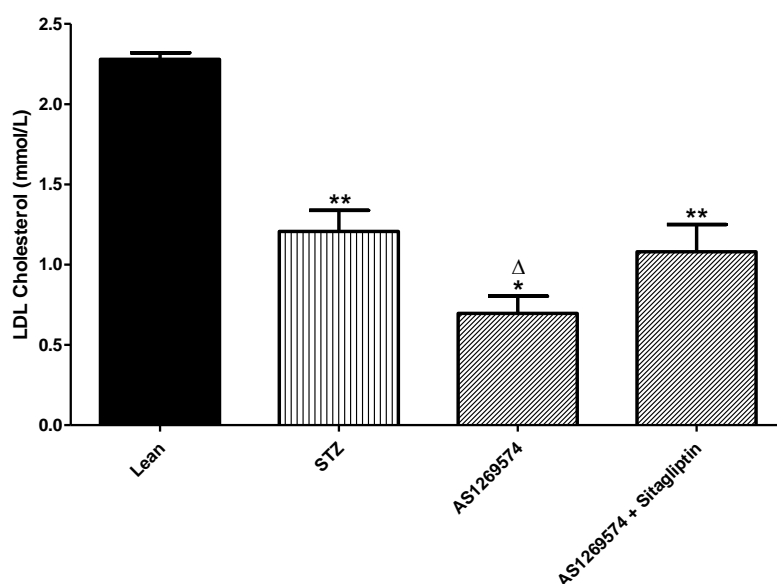
B.



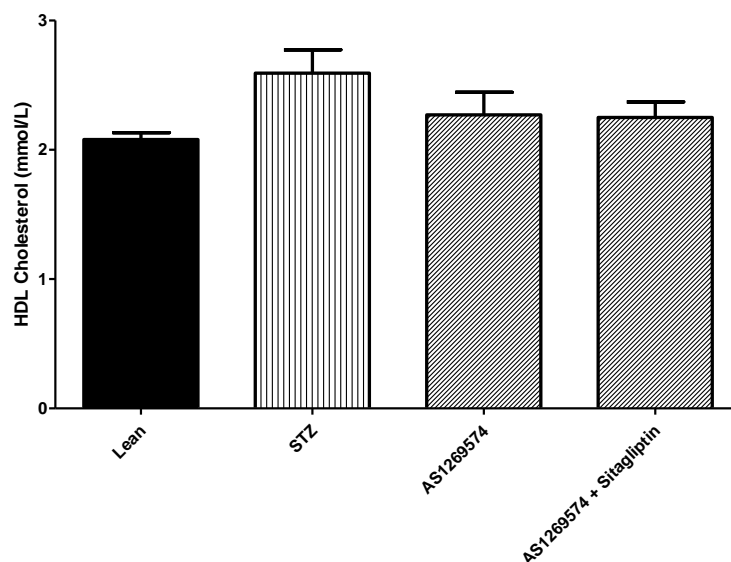
Effect of daily oral administration of saline (0.9%), AS1269574 monotherapy (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) combination therapy with Sitagliptin on (A) triglycerides and (B) total cholesterol following 21 day treatment period in streptozotocin-induced diabetic mice. Values are mean \pm SEM (n=6). * p<0.05 compared to the lean group. Δ p<0.05, $\Delta\Delta$ p<0.01 compared to the streptozotocin group.

Figure 7.53: Effects of long term daily oral treatment of GPR119 agonist AS1269574 on LDL and HDL cholesterol in multiple low dose streptozotocin mice

A.



B.



Effect of daily oral administration of saline (0.9%), AS1269574 monotherapy (0.1 μ mol/kg body weight) and AS1269574 (0.1 μ mol/kg body weight) combination therapy with Sitagliptin on (A) HDL cholesterol and (B) LDL cholesterol following 21 day treatment period in streptozotocin-induced diabetic mice. LDL was calculated using the Friedewald equation (LDL cholesterol = Total cholesterol – HDL cholesterol – (Triglycerides/5)). Values are mean \pm SEM (n=6). * $p < 0.05$ and ** $p < 0.01$ compared to lean mice. Δ $p < 0.05$ compared to streptozotocin treated mice.

Chapter 8

General Discussion

8.1: GPCR-based therapies

There are over 860 GPCRs present in the human genome which are expressed at low levels throughout the body (Fredriksson, Schioth 2005). GPCRs are of particular interest with 36% of FDA approved drugs targeting GPCRs (Rask-Andersen, Almen & Schioth 2011). Rhodopsin-like Class A receptors are highly targeted as they are the most prevalent GPCR superfamily in a range of genomes (Fredriksson, Schioth 2005). There is further pharmaceutical interest in the identification of novel GPCRs as well as new GPCR ligands as only 10% of currently identified GPCRs are targeted by drugs (Garland 2013). Therefore a large number of GPCRs may still be targeted for therapies for a range of syndromes.

Over 150 GPCRs are described as orphan receptors with no identified ligands (Ngo *et al.* 2016). The identification of endogenous and synthetic GPCR ligands requires better understanding of the physiological, pharmacological and pathological properties of these receptors. Advances in receptor overexpression or artificially expressed receptor technologies as well as *in vitro* and *in vivo* knockout technologies have helped to identify new GPCR ligands (Davenport *et al.* 2013). The identification and utilisation of novel GPCR ligands is also an area of research for new therapies for a range of diseases.

Type 2 Diabetes is usually treated with an initial prescription of metformin followed by metformin in combination with a sulphonylurea and finally insulin after the progressive loss of pancreatic β -cell mass (Matthews, Wallace 2005, Bailey, Turner 1996). However, some of the currently approved therapies include the targeting of the GLP1R with both exenatide and liraglutide (Kendall *et al.* 2005, Buse *et al.* 2010, Shyangdan *et al.* 2011). The majority of GPCRs targeted by drugs are Class A rhodopsin-like receptors, however GLP1R is a Class B receptor (de Graaf *et al.* 2016). Another GPCR of interest in the treatment of Type 2 Diabetes is GIPR. The insulinotropic effect of GIP in individuals with Type 2 Diabetes is diminished (Nauck *et al.* 1993). However when individuals with Type 2 Diabetes were administered a bolus injection of GIP, the insulinotropic effect of GIP was observed (Meier *et al.* 2004) demonstrating that the GIPR on pancreatic β -cells can be activated and targeted in the treatment of Type 2 Diabetes. This is further confirmed as the insulinotropic effect of GIP returns upon stabilisation of blood glucose to normal levels (Höjberg *et al.* 2009). In fact a novel long acting GIPR agonist known as AC163794 has been developed and this may have potential in the treatment of Type 2 Diabetes (Tatarkiewicz *et al.* 2013). Finally, while the GIP effect on insulin secretion is diminished in individuals with Type 2 Diabetes, GIPR knockout prevented weight gain, reduced insulin resistance and adiposity and antagonistic targeting of this receptor may be a viable therapeutic option in the treatment of Type 2 Diabetes (Irwin, Flatt 2009).

The receptors for incretin hormones are not the only GPCRs which have been targeted for the treatment of Type 2 Diabetes. One receptor which has generated significant interest is GPR40. This receptor was targeted due to its ability to increase glucose-stimulated insulin secretion upon activation by long chain fatty acids (Itoh *et al.* 2003b). A number of GPR40 agonists have been used in clinical trials with fasiglifam reaching stage 3 clinical trials but these were stopped due to concerns over liver toxicity (Li *et al.* 2015). Other GPR40 agonists which have undergone clinical trials as possible Type 2 Diabetes therapies include JTT-851, which has undergone phase 2 clinical trials (Watterson *et al.* 2014), ASP5034, LY2881835 and AMG837 all of which have been used in clinical trials but have not progressed past phase 1 (Houze *et al.* 2012, Krasavin *et al.* 2016). Further research into GPR40 agonists could include agonists which elicit an incretin response such as AM-1638 and AM-6226 possibly in combination with a DPP-IV inhibitor (Luo *et al.* 2012). GPR120 is also activated by long chain fatty acids however it has not received the same interest as GPR40 as its expression in β -cells has been questioned and it has reduced overall expression in the pancreas compared to GPR40 (Amisten *et al.* 2013, Stone *et al.* 2014). Previous research in our lab has shown that GPR120 activation has an insulinotropic effect on the pancreas (Moran *et al.* 2014b). The presence of GPR120 has been well established in the L and K cells of the intestine and may have effects on the incretin hormones as well as other endocrine hormones of the intestine (Reimann, Gribble 2015). The presence of GPR120 in the endocrine cells of the intestine and pancreas make it an interesting target for the treatment of Type 2 Diabetes. Another fatty acid receptor which has been used in clinical trials for the treatment of Type 2 Diabetes is GPR119. Within healthy human volunteers the GPR119 agonist JNJ-38431055 demonstrated an ability to increase incretin and PYY secretion after a single dose and exhibited insulinotropic ability upon infusion (Katz *et al.* 2011). However in individuals with Type 2 Diabetes these effects were ablated (Katz *et al.* 2012). Other GPR119 agonists which have been used in clinical trials include APD-597, MBX-2982, GSK1292263 and PSN-821 with most agonists making it to phase 2 trials (Cornall *et al.* 2013, Witkamp 2010). GSK1292263 showed no effects on circulating glucose or insulin but increased circulating PYY which mean that it may play a role in the reduction of obesity and other metabolic disorders (Nunez *et al.* 2014b). Further understanding of GLP1R and GIPR signalling may be required to utilise this agonist as a diabetic therapy due to its incretin secreting ability. A final GPCR which has been targeted for the treatment of Type 2 Diabetes is TGR5 due to the ability of this receptor to increase GLP-1 secretion upon activation (Duan *et al.* 2015). The presence of this receptor in the heart and gallbladder has led to severe side effects and it has been less extensively studied as a result of these.

8.2: GPCRs activated by peptides

The work described in Chapter 3 in this thesis outlines how a GPCR present in the β -cells of the pancreas can be activated by a peptide resulting in increased insulin secretion. One of the main complications in Type 2 Diabetes is the control of hyperglycaemia and a direct effect of GPCR agonists on the pancreas will lead to a reduction in circulating glucose as observed when administering sulphonylureas or GLP1R agonists. The use of a GPCRs targeted by peptides as a possible therapy for Type 2 Diabetes is supported by the evidence described above. Activation of GPR75 by CCL5 led to increased insulin secretion both *in vitro* and *in vivo*. Direct binding studies have not been able to prove the association between GPR75 and CCL5 (Southern *et al.* 2013) however the work carried out in this study and others have provided evidence that CCL5 activates GPR75 (Ignatov *et al.* 2006, Liu *et al.* 2013).

CCL5 increased insulin secretion confirming results seen previously (Liu *et al.* 2013). CCL5 was shown to be expressed in the β -cells of the pancreas in both lean and HFF mice providing evidence that the insulintropic effect of this agonist is through direct action on GPR75. The increased expression of this receptor in HFF mice make it an attractive therapeutic target due to the close relationship between Type 2 Diabetes and obesity, however these results will need to be confirmed in human cell lines and subjects. While previous work has demonstrated an effect of CLL5 on the pancreas, this study is the first study to identify the presence of GPR75 in the enteroendocrine cells of the intestine. Therefore this agonist may have potential as an oral anti-diabetic therapy. Both oral and IP administration of CCL5 elucidated an insulintropic response *in vivo*. The results in this study demonstrate the suitability of GPR75 activation as a potential therapy for the treatment of Type 2 Diabetes.

8.3: GPCRs activated by monatomic ions

GPCRs have a range of ligands including ions (Kobilka 2006) which are unable to enter the cell through the tightly bound seven transmembrane domain configuration of these receptors (Ji, Grossmann & Ji 1998). Previous research has shown that activation of GPCR with monatomic ions has an insulintropic effect *in vitro* and *in vivo* (Moran *et al.* 2016b). This study identified the effects of GPR39 activation on intestinal hormone secretion. The only GPR39 agonist which demonstrated a secretory effect on GLP-1 *in vitro* was CuCl_2 while all agonists tested demonstrated an effect on GIP secretion. This was confirmed with immunocytochemistry which failed to show GPR39 in GLP-1 secreting cells, however it was found to be present in both PYY and GIP secreting cells. Both PYY and GLP-1 are secreted from the L cells of the intestine, therefore activation of GPR39 may still lead to GLP-1 secretion. This was confirmed with *in vivo* studies which showed both CuCl_2 and ZnCl_2 increased GLP-1 and GIP secretion confirming previous research demonstrating that oral

administration of GPR39 agonists reduced blood glucose and increased insulin secretion (*Moran et al.* 2016b). Only one GPR39 agonist which increased GLP-1 secretion has been previously described (Peukert *et al.* 2014) and the research carried out in this study demonstrates two more GPR39 agonists with this action. This is also the first study to assess the ability of these agonists to increase incretin hormone secretion as well as being the first study to assess the acute effects of GPR39 agonists in combination with Sitagliptin *in vivo*.

8.4: GPCRs activated by fatty acids

Previous work has shown that GPR55, GPR119 and GPR120 can be activated by fatty acids of varying lengths and that this can lead to increased insulin secretion from the pancreas (McKillop *et al.* 2013, McKillop *et al.* 2016, Moran *et al.* 2014a, Moran *et al.* 2014b). Chapter 5 confirmed the insulinotropic effect observed when GPR120 is activated in the pancreas. GPR120 was found to be expressed in the small intestine (Hirasawa *et al.* 2005a) and the work carried out in Chapter 5 describes the expression of GPR120 in both the L and K cells of the small intestine. Both GPR120 and GPR40 can be activated by long chain fatty acids, however studies using si and shRNA has demonstrated that the agonists used in the studies performed in Chapter 5 are specific for GPR120 (Hirasawa *et al.* 2005, Tanaka *et al.* 2008). With the expression of GPR120 in both the pancreas and small intestine established, the study carried out in Chapter 5 determined that GPR120 activation by long chain fatty acids resulted in increased insulin and incretin secretion *in vitro*.

GW9508 was the only GPR120 agonist to increase both GLP-1 and GIP secretion *in vitro* while both ALA and DHA increased GLP-1 secretion. For this reason GW9508 was chosen for further *in vivo* analysis along with DHA. Previously, intraperitoneal administration of GPR120 agonists has demonstrated their direct effect on the pancreas (Moran *et al.* 2014b). In order to assess the effects of GPR120 agonists on incretin secretion, GPR120 agonists were administered *in vivo* via oral gavage. Agonists were also administered with the DPP-IV inhibitor Sitagliptin to try and prolong the effects of these hormones. Both agonists tested demonstrated the ability to increase insulin, GLP-1 and GIP secretion *in vivo*. Both DHA and GW9508 in combination with Sitagliptin reduced appetite. Overall these results show that GPR120 can be activated in both the intestine and pancreas and may be targeted for the regulation of glucose homeostasis.

Along with the previous research showing that GPR55 may be activated directly in the pancreas to increase insulin secretion, work carried out in incretin receptor knockout mice suggests that GPR55 activation may play a role in incretin hormone secretion (McKillop *et al.* 2016). The work carried out in Chapter 6 was designed to assess the ability of GPR55 agonists to increase intestinal hormone secretion both *in vitro* and *in vivo*. The only previously work in this area studied the effect of GPR55 agonist LPI on GLP-1 secretion while there are no currently published studies which have looked

at direct GPR55 activation on GIP secretion. GPR55 is an interesting target for metabolic disorders as it has been described as a putative cannabinoid receptor and may be used to reduce appetite while it is also a fat sensing receptor like GPR119, GPR120 and GPR40 (Henstridge, Brown & Waldhoer 2016). The work described in Chapter 6 confirmed the previously described effects of GPR55 activation on pancreatic β -cells. GPR55 has previously been shown to be present in the L-cells of the intestine (Harada *et al.* 2017) however this is the first study to describe the presence of GPR55 in the K cells of the intestine. It is also the first study to describe the ability of Abn-CBD, OEA and PEA to activate GPR55 and increase GLP-1 and GIP secretion *in vitro*. The presence of GPR55 in the L cells of the intestine was further confirmed as it is co-localised with PYY and this is the first study to describe this.

Previous work used intraperitoneal injection of GPR55 agonists to demonstrate their effect *in vivo* while oral administration of these agonists in incretin receptor knockout mice demonstrated a possible incretin effect (Moran *et al.* 2014b, McKillop *et al.* 2016). The *in vivo* work carried out in Chapter 6 involved oral administration of GPR55 agonists to confirm this incretin effect. Agonists were administered in combination with Sitagliptin. Oral administration of Abn-CBD and AM251 confirmed the previously seen insulin stimulating effects while also describing the ability of these agonists to increase GLP-1 and GIP for the first time. Abn-CBD administration alone reduced appetite while AM251 administration in combination with Sitagliptin had a similar effect. The results described in Chapter 6 suggests that GPR55 can be targeted to help regulate the impaired glucose homeostasis seen in Type 2 Diabetes.

A direct effect of GPR119 in the β -cells has been described (Moran *et al.* 2014a, McKillop *et al.* 2016), however there is also evidence that the ability of GPR119 agonists to reduce blood glucose and increase insulin secretion is dependent on the incretin response (Panaro *et al.* 2017b). The expression of GPR119 in the endocrine cells of the intestine has previously been described (Nunez *et al.* 2014a, Parker *et al.* 2009) and the work described in Chapter 7 confirmed the presence of GPR119 in the K and L cells of the intestine. Knockout of GPR119 has shown to ablate the ability of GPR119 agonists to increase GLP-1 secretion (Moss *et al.* 2015) and the study which was conducted confirmed the ability of GPR119 agonists to increase GLP-1 secretion *in vitro*. The effects of GPR119 agonists on GIP secretion was also described *in vitro* and these results were in agreement with the effects of GPR119 activation which have previously been published (Flock *et al.* 2011, Patel *et al.* 2014, Chu *et al.* 2008).

Previous *in vivo* work has shown that GPR119 agonists have a direct effect on the pancreas (Moran *et al.* 2014a). Later work in incretin receptor knockout mice suggested that GPR119 agonists may have the ability to increase incretin hormone secretion *in vivo* (McKillop *et al.* 2016). Therefore the *in vivo* studies carried out in Chapter 7 involved oral administration of GPR119 agonists so that the

effects of these agonists on incretin secretion could be assessed directly. Both AS1269574 and OEA increased insulin secretion, reduced blood glucose and increased incretin hormone secretion. These agonists were administered in combination with Sitagliptin which helped to further reduce blood glucose. Both agonists in combination with Sitagliptin reduced feeding. Long term administration of AS1269574 mirrored these effects.

Overall, the results from the studies described above show that fatty acid agonists can be used to target GPCRs in the intestine and the pancreas to regulate glucose homeostasis through a variety of mechanisms and thus have potential as a therapy for the treatment of Type 2 Diabetes.

8.5 Conclusions and Limitations of the studies described in this thesis

The work described in this thesis leads to the following conclusions:

- GPCRs are present in the gastrointestinal tract and can be activated by a range of fatty acid, peptide based and monoatomic ion based agonists
- Activation of gastrointestinal GPCRs can increase GLP-1 and GIP secretion and this is achieved through a range of GPCRs as well as a range of agonists
- GPCR agonists can also have a direct effect on the pancreas and directly stimulate insulin secretion
- Long term oral administration of fatty acid based agonists can improve glucose homeostasis and demonstrates the potential of GPCR activation for the treatment of Type 2 Diabetes

The work described in this thesis also has several limitations. These are as follows:

- There are no direct binding studies performed to demonstrate agonist specificity for the GPCR being targeted
- Some agonists target a number of GPCRs
- siRNA knockdown was not used and this would have helped to determine agonist specificity
- While the studies discussed in this thesis were being performed, the CRISPR Cas9 knockout system was described and this would be a useful tool in the future to confirm agonist specificity

8.6: Mode of action of GPCRs studied in this thesis

Previous research has shown that the GPCRs studied in this thesis may regulate glucose homeostasis through direct action on the pancreas, however work in incretin receptor knockout mice suggests

that these GPCRs play a role in incretin hormone secretion as well as (McKillop *et al.* 2013, Liu *et al.* 2013, Moran *et al.* 2014b, Moran *et al.* 2014a, Moran *et al.* 2016b, McKillop *et al.* 2016). The work described in this thesis demonstrates a direct action of GPCR agonists on the enteroendocrine cells of the intestine. The ability of these receptors to increase intestinal and pancreatic hormone secretion and their role in glucose homeostasis are seen in Figure 8.1. Activation of intestinal GPCRs increases GLP-1 secretion which increases satiety due to its ability to cross the blood brain barrier. The brain returns a signal to the intestine to slow gastric emptying. Increased GLP-1 will also activate the GLP1R in the pancreas resulting in increased insulin secretion. GPCR agonists also have an insulinitropic effect through direct action on the GPCRs present in the pancreatic endocrine cells. Increased GLP-1 will also help to increase the β -cell mass of the pancreas. Increased GIP secretion from intestinal K cells will also demonstrate an insulinitropic effect in individuals without diabetes. The result of this process is a reduction in body weight, increased glucose tolerance and increased glucose sensitivity.

Table 8.1:

Agonist	Glucose Concentration Agonist was tested in	Target GPCR	Concentration that increases Insulin Secretion; significance of increase	Glucose Concentration Agonist was tested in	Concentration that increases GLP-1 Secretion; significance of increase	Concentration that increases GIP Secretion; significance of increase
CCL5	5.6mM	GPR75	10^{-9} to 10^{-5} M; * to***	2mM	??	??
	16.7mM		10^{-8} to 10^{-5} M; ** to***			
CuCl ₂	5.6mM	GPR39	15.6 to 500μM; ***	2mM	31.25 to 125μM; *	31.25 to 125μM; * to**
	16.7mM		15.6 to 500μM; ** to***			
NiSO ₄	5.6mM	GPR39	31.3 to 500μM; ***	2mM	No increase	31.25 to 125μM; * to**
	16.7mM		31.3 to 500μM; * to***			
ZnCl ₂	5.6mM	GPR39	15.6 to 500μM; ** to***	2mM	No increase	31.25 to 125μM; **
	16.7mM		15.6 to 500μM; ***			
Abn-CBD	5.6mM	GPR55	10^{-9} to 10^{-4} M; * to***	2mM	10^{-8} to 10^{-4} M; *	No increase
	16.7mM		10^{-9} to 10^{-4} M; * to***			
AM251	5.6mM	GPR55	10^{-8} to 10^{-4} M; ** to***	2mM	10^{-8} to 10^{-6} M; ** to***	No increase

	16.7mM		10^{-7} to 10^{-4} M; * to ***			
OEA	5.6mM	GPR55/ GPR119	10^{-8} to 10^{-4} M; * to ***	2mM	10^{-6} to 10^{-4} M; ***	10^{-6} to 10^{-4} M; * to ***
	16.7mM		10^{-10} to 10^{-4} M; * to ***			
PEA	5.6mM	GPR55/ GPR119	10^{-8} to 10^{-4} M; * to ***	2mM	10^{-8} to 10^{-4} M; ** to ***	10^{-6} to 10^{-4} M; * to ***
	16.7mM		10^{-8} to 10^{-4} M; * to ***			
AS1269574	5.6mM	GPR119	10^{-8} to 10^{-4} M; ** to ***	2mM	10^{-6} M; **	No increase
	16.7mM		10^{-8} to 10^{-4} M ** to ***			
ALA	5.6mM	GPR120	10^{-8} to 10^{-4} M; * to ***	2mM	10^{-8} to 10^{-6} M; **	No increase
	16.7mM		10^{-8} to 10^{-4} M; * to ***			
DHA	5.6mM	GPR120	10^{-9} to 10^{-4} M; ** to ***	2mM	10^{-8} to 10^{-6} M; ** to ***	No increase
	16.7mM		10^{-9} to 10^{-4} M; * to ***			
GW9508	5.6mM	GPR120	10^{-10} to 10^{-4} M; * to ***	2mM	10^{-6} M; ***	10^{-6} M; *
	16.7mM		10^{-9} to 10^{-4} M; * to ***			

The *in vitro* effects of the GPCR agonists used in this study on GLP-1, GIP and insulin secretion. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

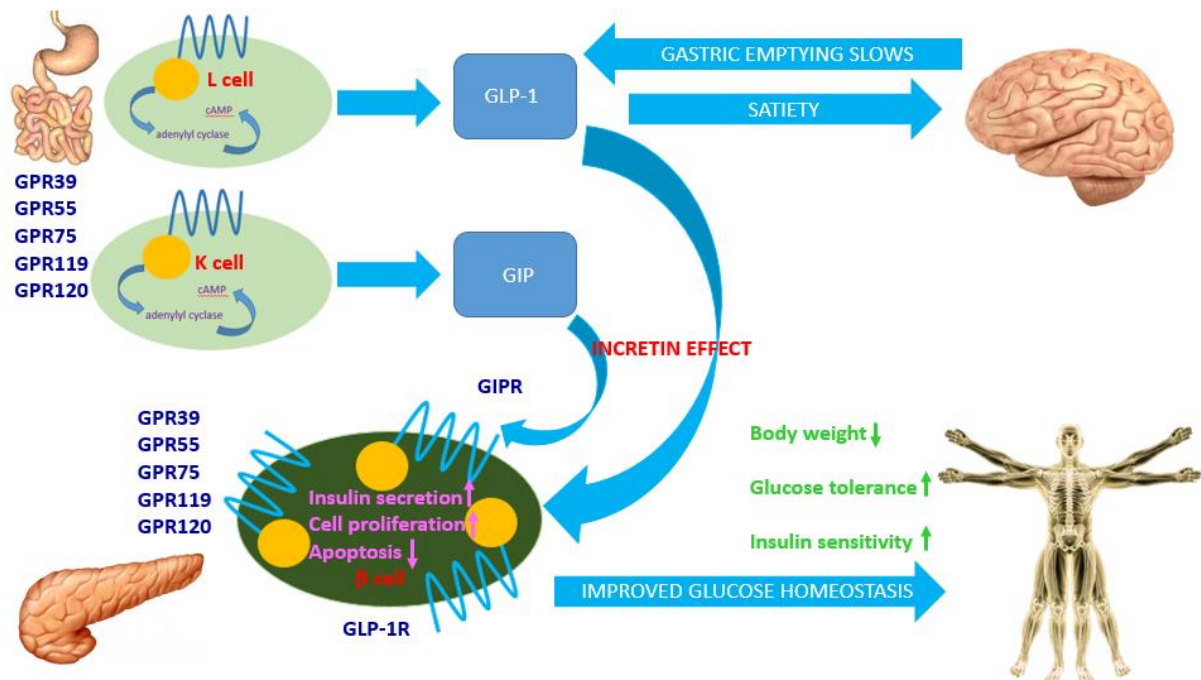
Table 8.2

Agonist	Admin.	Target GPCR	Time point Insulin Secretion is increased after administration,; significance of increase	Administration Method	Time point GLP-1 Secretion is increased after administration,; significance of increase	Time point GIP Secretion is increased after administration,; significance of increase
CCL5	Oral	GPR75	30 to 60 mins; * to ***	Oral	??	??
	IP		15 to 30 mins; * to ***			
CuCl ₂	Oral	GPR39	30 mins; ***	Oral	15 to 30 mins; ***	15 mins; ***
ZnCl ₂	Oral	GPR39	30 mins; ***	Oral	30 mins; ***	15 to 30 mins; ** to ***
Abn-CBD	Oral	GPR55	15 to 30 mins; * to ***	Oral	15 to 30 mins; ***	15 mins; ***
AM251	Oral	GPR55	15 to 30 mins; ** to ***	Oral	15 to 30 mins; ***	No increase
AS1269574	Oral	GPR119	30 mins; ***	Oral	15 to 30 mins; ***	15 mins; ***
OEA	Oral	GPR119	30 mins; ***	Oral	30 mins; ***	15 mins; ***
DHA	Oral	GPR120	30 mins; ***	Oral	30 mins; ***	15 mins; ***

GW9508	Oral	GPR120	30 mins; ***	Oral	15 to 30 mins; ***	15 mins; ***
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The *in vivo* effects of the GPCR agonists used in this study on GLP-1, GIP and insulin secretion. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 8.1 Mechanism of action of intestinal and pancreatic GPCRs as well as the incretin hormones, GLP-1 and GIP



8.7: Limitations and complications of currently approved therapies for Type 2 Diabetes

Many of the current therapies used in the treatment of Type 2 Diabetes are able to regulate glucose homeostasis in diabetic individuals, however they are unable to prevent the macrovascular complications which are caused by the associated chronic obesity (Cade 2008). If left untreated, these macrovascular complications will result in increased risk of mortality (Panzram 1987) however these effects on both mortality and morbidity can be reduced when therapies focus on regulation of blood pressure rather than hyperglycaemia (King, Peacock & Donnelly 1999). Intensive anti hyperglycaemic therapies have only been shown to reduce microvascular complications but have no effect on the macrovascular complications and individuals with hypertension were heavier and had higher plasma triglyceride levels (Diabetes Control and Complications Trial Research Group *et al.* 1993). The first generation of sulphonylurea therapies were also linked to increased mortality due to adverse cardiovascular events which may be due to the inhibition of vasodilation due to ischemia (Ashford *et al.* 1994). This was similar to the observed effects of the first generation of biguanide therapies which increased mortality due to adverse cardiovascular events. Administration of these biguanides also conferred an increased risk of lactic acidosis as discussed in Chapter 1. The inability of hypertension therapies to correct hyperglycaemia and the inability of hyperglycaemic medication to correct hypertension mean that one of the main challenges in antidiabetic therapies is the development of treatments which can reduce both the

microvascular and macrovascular complications which occur in people with Type 2 Diabetes due to the combined effects of hyperglycaemia and hypertension.

Treatment of hyperglycaemia in individuals with Type 2 Diabetes reduces the microvascular complications of the disease with the biggest reductions seen in surgeries to reduce complications of diabetic retinopathy (King, Peacock & Donnelly 1999). A study comparing the main treatments for hyperglycaemia compared sulfonylurea and insulin therapy to metformin and found that obese people administered metformin had fewer complications such as hyperglycaemia and weight gain. This is one of the many reasons why metformin is normally the first therapy used in the treatment of Type 2 Diabetes over other available therapeutics. The differences between metformin and other therapies may be due to the differing mechanisms through which metformin reduces hyperglycaemia. Despite being the first choice drug for the treatment of Type 2 Diabetes, there are still instances when administration of metformin is not recommended. Individuals with impaired kidney or liver function would not be suitable for treatment with metformin (Bailey, Turner 1996). While these drugs may reduce hyperglycaemia, their effect diminishes over time due to the destruction of pancreatic β -cells (Matthews, Wallace 2005, Bailey, Turner 1996). The progressive loss of β -cell mass means that all Type 2 Diabetes sufferers will end up on insulin therapy after having the disease for a long period of time. The limitations of antidiabetic and antihypertensive medications mean that affected individuals need to be prescribed various medications which will likely lead to lower compliance with prescribed guidelines. Therefore there is a need to develop new Type 2 Diabetes therapeutics which can reduce both hyperglycaemia and hypertension and which will prevent the progressive loss of pancreatic β -cell mass. The UKPDS has indicated that beta cell dysfunction begins at an early stage, prior to changes in blood glucose. Therefore better therapies for Type 2 Diabetes are required to tackle beta cell dysfunction. The ideal therapy would prevent diabetes and obesity due to the complications of hyperglycaemia and hypertension which are present in both of these syndromes. This therapy could be administered before the onset of β -cell dysfunction.

8.8: Future research

Previous research has described the presence of 293 GPCRs in the pancreas of mice (Amisten *et al.* 2013). These receptors have been further compared to those present in the human pancreas (Amisten *et al.* 2017). The identification of the different GPCRs in both humans and mice will be beneficial in future research for the identification of appropriate *in vivo* models as well as the identification of appropriate GPCR targets that are present in the human genome. In order to further understand the effect of GPCRs in the intestine the GPCRs present in the intestine would need to be identified, as seen with the pancreas. This presents its own challenges due to the differences between the main

sections of the intestine as well as the variety of cells scattered throughout the intestine, however *ex vivo* methods such as intestinal organoids (Fatehullah, Tan & Barker 2016) may be utilised to try and limit these to cells solely with an open confirmation and access to the lumen of the intestine. Furthermore there are a wide variety of hormones secreted from a wide variety of enteroendocrine cells in the intestine and the effects of GPCR activation on hormones such as PYY, CCK, ghrelin, gastrin and obestatin need to be further understood in order to further understand how receptors in the intestine can be targeted for the regulation of glucose homeostasis.

Much of the research described above has looked at the acute effects of GPCR activation except for the chronic effects on Abn-CBD and AS1269574 described in Chapters 6 and 7. Chronic treatment of these agonists has been described previously (McKillop *et al.* 2016), however this is one of the first studies to assess chronic administration of GPCR agonists in combination with Sitagliptin in order to try and better utilise the incretin effect. Previous chronic studies have been carried out for GPR40 and GPR119 (Li *et al.* 2015, Katz *et al.* 2011, Katz *et al.* 2012) but there are not many published chronic studies which combine GPCR agonists with other drugs for Type 2 Diabetes. There are still a lot of GPCR agonists which are yet to be identified and of those which have been identified there are still questions regarding long term administration which need to be answered.

One of the most important questions regarding research into GPCRs is surrounding the specificity of agonists for the receptor being studied. Previous studies include direct binding studies as well as blocking of the receptor with antagonists as well as knockdown with siRNAs. With the development of *in vitro* knockout technologies such as CRISPR (Zhang, Wen & Guo 2014) it is now possible to knockout the receptor to assess ligand binding. These techniques now allow researchers to assess the specificity of agonists for receptors before moving to *in vivo* models and will likely be a large area of research for those interested in GPCRs.

As described previously in this thesis, one of the main complications in Type 2 Diabetes is cardiovascular events caused by hypertension. A possible area of research would be to identify GPCRs which can help reduce hypertension and regulate glucose homeostasis at the same time. It would also be beneficial if GPCRs which can help to reduce inflammation are identified as this is another complication of Type 2 Diabetes. The difficulties with the current therapies for Type 2 Diabetes have also been described and these difficulties mean that there is a need to develop better therapies. The work described in this thesis describes how GPCRs can be targeted to help regulate glucose homeostasis through intestinal hormone secretion. GPCRs have already shown the potential to treat diabetes with the currently available Liraglutide and Exenatide. Fatty acid GPCRs have been further studied in clinical trials and the identification of new receptors or agonists for these receptors may provide a potential solution whether that be in a monotherapy or a combination therapy with Sitagliptin.

Chapter 9

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